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Regulation of the actin cytoskeleton by Ephrin-B signalling

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Abstract

Ephrin ligands and their Eph receptors play an essential role in angiogenesis during development. Both ephrins and Eph receptors are membrane-tethered proteins and their interaction at sites of cell-cell contact triggers bi-directional signalling, with signals transduced from the receptor (forward signalling) and ligand (reverse signalling). I have used two model systems to study ephrin-B2 signalling: Swiss 3T3 fibroblasts expressing exogenous ephrin-B2 and Human Umbilical Arterial Endothelial Cells (HUAECs) endogenously expressing ephrin-Bs. Stimulation of ephrin-B2 with soluble EphB receptors, has enabled the characterisation of the cellular responses, and signalling pathways, triggered by ephrin-B2 activation.

I have shown that clustering of expressed ephrin-B2 in cultured fibroblasts induces a loss of cell-cell contact, dependent on the presence of serum factors and independent of actin-myosin contractility. The intracellular domain of ephrin-B2 is essential: tyrosine phosphorylation of the ligand via Src, and binding of the adaptor protein Grb4 are required for loss of cell-cell contact.

Stimulation of endogenous ephrin-B2 in cultured endothelial cells results in dramatic cell retraction, and in a proportion of cells membrane blebbing. I have shown that the small GTPase Rho and activation of its downstream effector ROCK are essential for membrane retraction to occur, which is driven by an actin-myosin contraction event. In addition, I find that the c-Jun amino terminal kinase (JNK) is required for retraction, acting upstream of Rho/ROCK, and retraction occurs independently of Grb4. The cell contraction response to ephrin-B2 activation is rapid and transient with cells recovering to re-spread lamellipodia within minutes. Re-spreading is coupled to a loss of actin stress fibres and concomitant with down regulation of Rho and ROCK activity.

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Abbreviations

ADAM	A disintegrin and metalloprotease
AP	Anterior-posterior
APS	Ammonium persulphate
Arp	Actin related protein
bFGF	Basic fibroblast growth factor
BHK	Baby hamster kidney
BSA	Bovine serum albumin
CALI	Chromophore assisted laser inactivation
CAP	Cyclase associated protein
CLD1	Claudin-1
CLD4	Claudin-4
Con A	Concanavalin A
dH_2O	Distilled water
DAB	3,3'-diaminobenzidine
DMEM	Dulbecco's modified eagles medium
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
DV	Dorsal-ventral
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol-bis(beta-aminoethyl ether)-N,N,N',N'-tetraacetic acid
EGF	Epidermal growth factor
Eph	Erythropoietin-producing hepatocellular carcinoma cell line
F-actin	Filamentous actin
FAK	Focal adhesion kinase
FCS	Foetal calf serum
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor
FITC	Fluorescein isothiocyanate
GAP	GTPase activating proteins
GDI	Guanine nucleotide dissociation inhibitor
GDP	Guanine nucleotide diphosphate
GEF	Guanine nucleotide exchange factor
GPI	Glycosyl-phosphatidyl-inositol

Grb	Growth factor receptor-bound protein
GRIP	Glucocorticoid receptor-interacting protein
GST	Gltathione-S-transferase
GTP	Guanine nucleotide triphosphate
HCC	Hepatocellular carcinoma cells
HEK	Human embryonic kidney
hnRNPK	Human heterogeneous nuclear ribonucleoprotein K
HRP	Horseradish peroxidase
HUAECs	Human Umbilical Arterial Endothelial Cells
HUVECs	Human Unbilical Venous Endothelial Cells
IBD	Inflammatory bowel disease
IPTG	isopropyl-β-D-thiogalactopyranoside
JNK	c-Jun amino terminal kinase
kDa	Kilo daltons
LM	Lateral-medial
LMW-PTP	Low molecular weight protein tyrosine phosphatase
MAP kinase	Mitogen activated protein kinase
MES	2-Morpholinoethanesulfonic acid
MLC	Myosin light chain
MLCP	Myosin light chain phosphatase
MYPT	Myosin phosphatase targeting subunit
NIK	Nck-interacting kinase
NMDA	*N*-methyl-$_D$-aspartate
N-WASP	Neural-WASP
OT	Optic tectum
PAGE	Polyacrylamide gel electrophoresis
PAK	p21 activated kinase
PBS	Phosphate buffered saline
PCP	Planar cell polarity
PDGF	Platelet derived growth factor
PDR	Proliferative diabetic retinopathy
PDZ	PSD-95/Dlg/ZO-1
PI3K	Phosphatidylinositol 3-kinase
PICK 1	Protein interacting with C-kinase-1

PHIP	Pleckstrin homology domain interacting protein
PMSF	Phenylmethylsulphonyl fluoride
PTP-BL	Protein tyrosine phosphatase basophil-like
PVDF	Polyvinylidene fluoride
RBD	Rhotekin Rho binding domain
RGC	Retinal ganglion cell
RGS	Regulator of G-protein signalling
ROCK	Rho kinase
ROP	Retinopathy of prematurity
RTK	Receptor tyrosine kinase
SAM	Sterile alpha motif
SC	Superior colliculus
SDF-1	Stromal-cell derived factor 1
SDS	Sodium dodecyl sulphate
SFM	Serum free medium
SH2	Src homology 2
SH3	Src homology 3
SHEP1	SH2 domain-containing Eph receptor-binding protein 1
TBS	Tris buffered saline
Tie-2	Tyrosine kinase with immunoglobulin-like and EGF-like domains 2
TN	Temporal-nasal
Toca-1	Transducer of Cdc42-dependent actin assembly
TRITC	Tetramethyl rhodamine isothiocyanate
TZ	Termination zones
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
WASP	Wiskott-aldrich syndrome protein
WAVE	WASP family Verprolin-homologous protein
WIP	WASP interacting protein

Chapter 1

Introduction

Cell migration and adhesion, involving precise cell movements, cell-reorganisation and intercellular communication, is critical throughout development to enable cells to reach their correct targets and ultimately for their correct function. Receptor tyrosine kinases are already known to play a role in many fundamental cell processes including cell migration, survival, proliferation, differentiation and cell cycle control. Co-ordinated changes in cell shape and motility form the basis of many of the events controlled by the family of receptor tyrosine kinases known as the Eph receptors and their ligands the ephrins, such that many of the intracellular signalling pathways initiated by Eph receptor-ephrin signalling ultimately regulate the actin cytoskeleton.

Ephrin ligands and their Eph receptors are membrane-tethered proteins known to regulate both repulsive and attractive interactions between contacting cells, and have been shown to function in a wide range of developmental processes. They are particularly important for the tissue patterning and morphogenesis events that give rise to a structured embryo. However, many of the emerging signalling pathways downstream from Eph receptor-ephrin interactions remain poorly characterised, as are the cellular events triggered by such signalling cascades.

1.1 Background

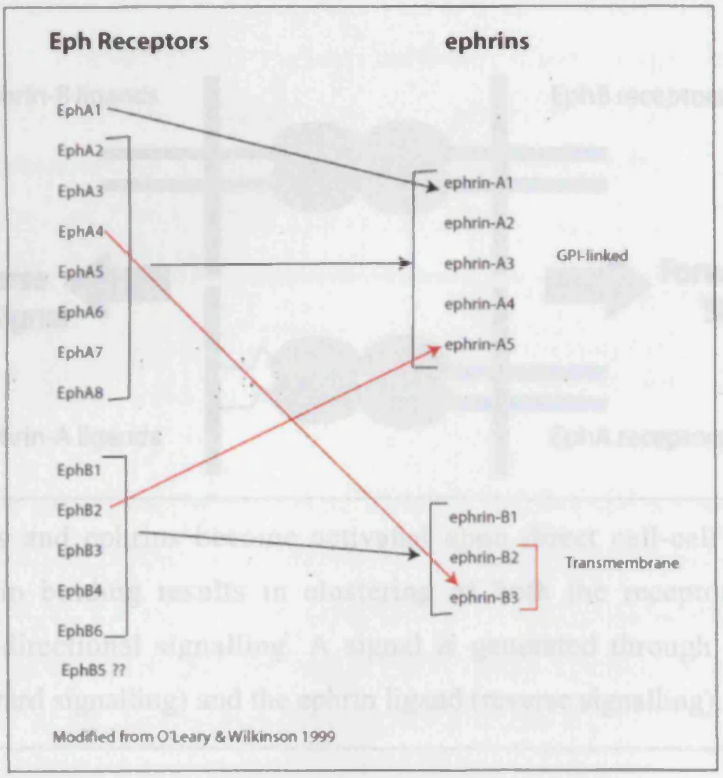
The first Eph receptor, EphA1, was discovered in 1987, through a screen for genes up-regulated in epithelial tumours, and termed Eph after the erythropoietin-producing hepatocellular carcinoma cell line from which it was cloned (Hirai et al., 1987). Subsequent members of the Eph receptor family were discovered from screens for tyrosine phosphorylated proteins and for tyrosine kinase domains (Letwin et al., 1988; Lindberg and Hunter, 1990; Pasquale and Singer, 1989). Eph receptors were initially described as 'orphan receptors', since they were without known ligands. However, in the mid 1990s proteins capable of binding their extracellular domains were identified (Bartley et al., 1994; Beckmann et al., 1994; Cheng and Flanagan, 1994; Fletcher et al., 1994; Shao et al., 1994). The first ligand, B61/ephrin-A1, had previously been isolated as a protein with unknown function (Holzman et al., 1990) but was later shown to be the ligand for Eck/EphA2 (Bartley et al., 1994).

The Eph receptor family is the largest known subfamily of receptor tyrosine kinases, and a similarly large number of ephrin ligands also exist. To date 16 receptors and 9 ligands have been identified in vertebrates, (14 receptors and 8 ligands in mammals) (Murai and Pasquale, 2003). Eph receptors have been identified in species as diverse as *Caenorhabditis elegans* (George et al., 1998), zebrafish (Xu et al., 1994), and *Drosophila melanogaster* (Scully et al., 1999). Much work, encompassing genetic, biochemical and cell biological studies, has been carried out over the last 15-20 years to determine their structure, localisation and function.

1.2 Eph receptor and ephrin subclasses

Ephrin ligands are divided into two subclasses based on their attachment to the membrane. The A class ephrins are attached to the outer leaflet of the cell membrane by a glycosyl-phosphatidyl-inositol (GPI) linkage, whereas the B class ephrins are transmembrane proteins (Gale et al., 1996b). Eph receptors are also divided into two subclasses, the EphAs and the EphBs, based on sequence homology and ligand binding affinity (Gale et al., 1996b). EphA receptors bind to ephrin-A ligands and EphB receptors bind to ephrin-B ligands. Binding within classes is known to be promiscuous, however interactions are thought to be restricted such that binding to members of another subclass does not occur. The exception to this is EphA4, which can bind to both ephrin-As and ephrin-Bs (Gale et al., 1996b). Although there is a high degree of promiscuity between Eph receptors and ephrins of the same class, they are unlikely to be functionally interchangeable. There are wide variations in binding affinities between different receptor-ligand pairs. For example, the only known ligand for EphB4 is ephrin-B2, where as EphB2 is known to bind ephrin-B1, ephrin-B2 and ephrin-B3 (Flanagan and Vanderhaeghen, 1998). Recently however it has been shown that ephrin-A5 is capable of binding to and activating the EphB2 receptor (Himanen et al., 2004). Signalling between subclasses may occur more readily than previously thought (Fig. 1.1).

Figure 1.1: Eph receptor and ephrin families and their binding partners

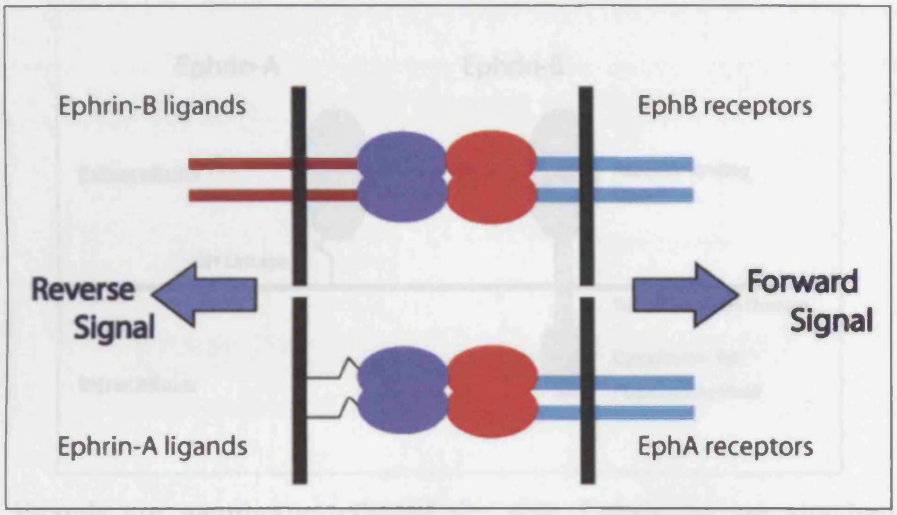


Ephrins are divided into two subclasses based on their attachment to the membrane. Two classes of Eph receptors also exist, with the division based on sequence homology and ligand binding affinity. Generally EphA receptors bind to ephrin-A ligands and EphB receptors bind to ephrin-B ligands (black arrows). However, instances of binding across subclasses have been shown to occur (red arrows). At present the ligands(s) for EphB5 are not known.

1.2.1 Structure of Eph receptors and ephrins

Both ephrins and Eph receptors are membrane-tethered proteins and therefore interactions between Eph receptors and ephrins require direct cell-cell contact. Signalling is bi-directional as, not only can a signal be transduced from the receptor-bearing cell (forward signalling) but the ephrin ligand also becomes activated and transmits a signal into its respective cell, known as reverse signalling (Fig. 1.2). Physiological roles for ephrin-B signalling have now been described (Davy and Soriano, 2005).

Figure 1.2: Bi-directional Signalling

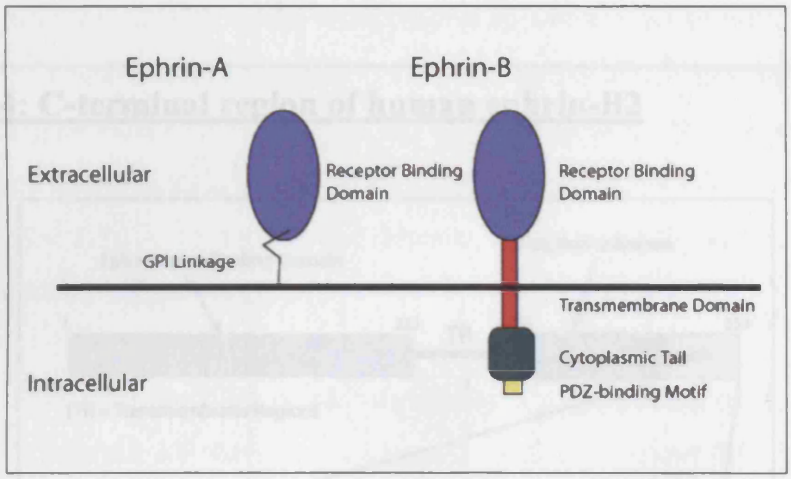


Eph receptors and ephrins become activated upon direct cell-cell contact. Eph receptor/ephrin binding results in clustering of both the receptor and ligand, triggering bi-directional signalling. A signal is generated through both the Eph receptor (forward signalling) and the ephrin ligand (reverse signalling).

1.2.1.1 Ephrins

There are three members of the ephrin-B subclass and they each possess an extracellular receptor-binding domain, a transmembrane fragment and cytoplasmic domain (Fig. 1.3). The crystal structures of both the ephrin-B1 and ephrin-B2 ectodomains have recently been elucidated (Nikolov et al., 2005; Toth et al., 2001), revealing the overall structure of ephrin-B1 to be similar to that of ephrin-B2. Although, two main differences are apparent, which affect ligand oligomerisation and receptor-ligand binding specificity (Nikolov et al., 2005). There are five members of the ephrin-A subclass (ephrin-A1-ephrin-A5). They each have an extracellular receptor-binding domain and are attached to the membrane via a GPI anchor. The ephrin-As do not posses an intracellular domain (Fig. 1.3).

Figure 1.3: Schematic representation of the structure of ephrins



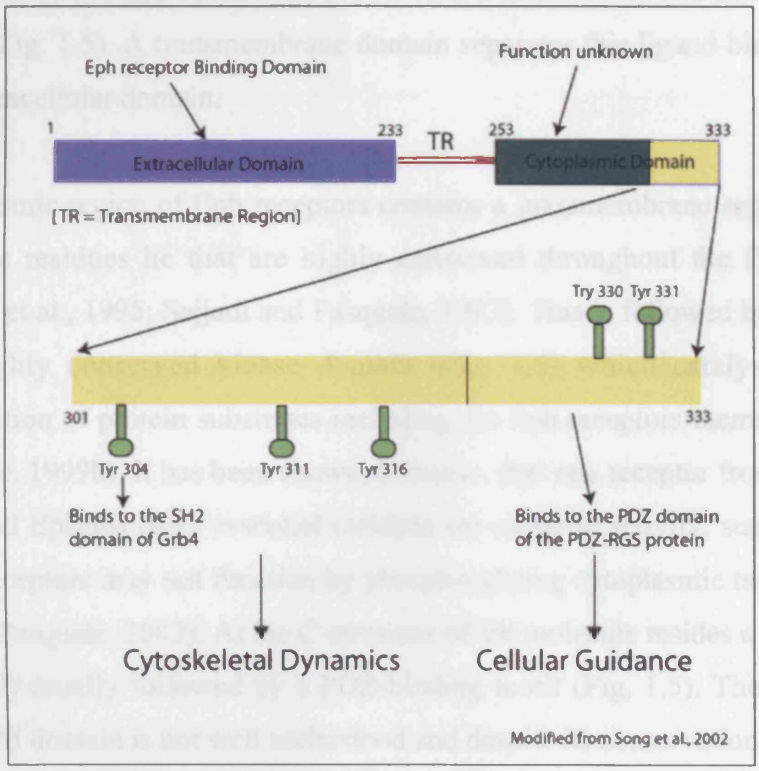
Ephrin ligands are membrane-tethered proteins. Ephrin-As are attached to the membrane via a GPI linkage and possess an extracellular receptor-binding region. Ephrin-Bs exist as transmembrane proteins. They also possess an extracellular domain responsible for receptor-binding, together with a cytoplasmic domain, which is important for signal transduction downstream of ephrin-Bs.

A striking feature of the ephrin-B ligands is their highly conserved carboxy-terminal tails, which are 90-100 residues in length. The last 33 amino acids are particularly conserved; across this region ephrin-B1 and ephrin-B2 are identical and only 3 amino acids differ in ephrin-B3. Interestingly, this region shows no detectable homology with other proteins, indicating its functional role in ephrin-B signalling (Song et al., 2002).

The last 33 residues of the carboxy-terminal region contain five functionally important tyrosine residues, three of which become phosphorylated upon ephrin clustering in response to receptor binding (Bruckner et al., 1997; Holland et al., 1996; Kalo et al., 2001). At the extreme carboxy terminus lies a PDZ binding domain (Lin et al., 1999). Numerous studies over the last few years support a functional role for the cytoplasmic domain in the propagation of the reverse signal. Downstream signalling events involved in cytoskeletal changes and cellular guidance result from the binding of PDZ domain proteins as well as tyrosine phosphorylation of ephrin-Bs

and consequent binding of adaptor proteins (Fig. 1.4) (Bruckner et al., 1999; Cowan and Henkemeyer, 2001; Lu et al., 2001).

Figure 1.4: C-terminal region of human ephrin-B2



Ephrin-Bs possess a highly conserved C-terminal tail, known to contain five functionally important tyrosine residues and a PDZ binding motif. Phosphorylation on tyrosine has been shown to be an important feature of ephrin-B signalling and numerous PDZ domain proteins have been shown to bind ephrin-Bs. Reverse signals from ephrin-Bs are known to regulate cytoskeletal dynamics and cellular guidance.

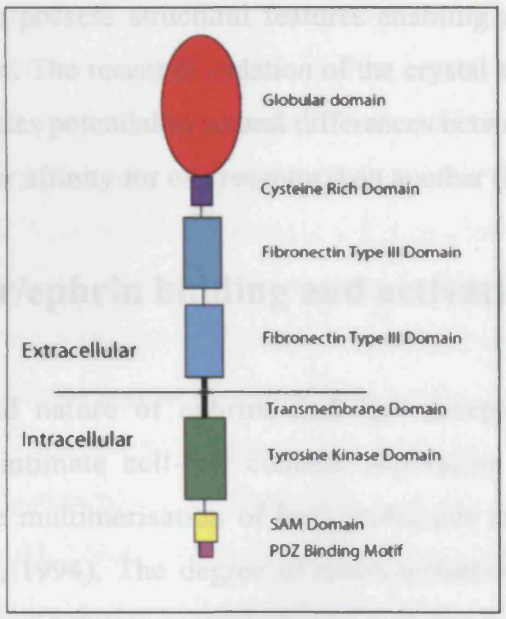
1.2.1.2 Eph receptors

Like all receptor tyrosine kinases, the Eph receptors are type I transmembrane proteins (van der Geer et al., 1994). Their extracellular region contains a highly conserved N-terminal globular domain (Fig. 1.5), which is necessary and sufficient for ligand recognition and binding (Labrador et al., 1997), and is structurally similar to the carbohydate-binding domain of lectins (Himanen et al., 1998). The crystal

structure of the extracellular, ligand-binding domain of EphB2 has been elucidated and shown to fold into a compact jelly roll β sandwich composed of 11 anti-parallel β strands (Himanen et al., 1998). Immediately adjacent is a cysteine rich region (containing an epidermal growth factor (EGF)-like motif), which may be involved in receptor-receptor interactions modulating oligomerisation often observed upon ligand binding (Boyd and Lackmann, 2001). This is followed by two fibronectin type III repeats (Fig. 1.5). A transmembrane domain separates this ligand-binding region from the intracellular domain.

The cytoplasmic region of Eph receptors contains a juxtamembrane segment where two tyrosine residues lie that are highly conserved throughout the Eph receptor family (Fox et al., 1995; Sajjadi and Pasquale, 1993). This is followed by a centrally located, highly conserved kinase domain (Fig. 1.5) which catalyses tyrosine phosphorylation of protein substrates including the Eph receptors themselves (Kalo and Pasquale, 1999b). It has been shown, however, that one receptor from each class (EphA10 and EphB6) lacks essential residues for catalytic activity, suggesting that these two receptors may not function by phosphorylating cytoplasmic target proteins (Murai and Pasquale, 2003). At the C-terminus of the molecule resides a sterile alpha motif (SAM) usually followed by a PDZ-binding motif (Fig. 1.5). The function of the Eph SAM domain is not well understood and despite its conservation, its removal does not disrupt Eph function (Boyd and Lackmann, 2001). Structural studies indicate that the SAM domain may modulate receptor dimerisation or clustering (Stapleton et al., 1999; Thanos et al., 1999), while other experiments suggest it may bind adaptor proteins (Boyd and Lackmann, 2001).

Figure 1.5 Structure of Eph receptors



Eph receptors are transmembrane proteins. The extracellular region is composed of a globular ligand-binding domain, a cysteine rich region followed by two fibronectin type III repeats. The intracellular region is composed of the tyrosine kinase domain, a sterile α motif (SAM) and a PDZ binding domain.

1.2.1.3 Structural features influencing receptor-ligand binding specificities

In vivo, many ephrins have overlapping expression patterns (Flenniken et al., 1996). Therefore differences in binding affinities are likely to be crucial where ligands are co-expressed (Feldheim et al., 2000). Mutational and chimeric analyses of the EphB2 ligand-binding domain identified an extended loop important for ligand binding and class specificity. This loop is conserved within a subclass, but not across subclasses and the length of the loop appears to confer class discrimination (Himanen et al., 1998). In addition, analyses of the sequence conservation of ephrin ectodomains has led to the proposal that a surface region consisting of a small concave hydrophobic pocket surrounded by charged amino acids represents a crucial site for receptor recognition and class specificity of ephrins. Varying degrees of amino acid

conservation provide a mechanism to influence receptor binding, while allowing some promiscuity within a class as seen *in vitro* (Toth et al., 2001). Both receptors and ligands appear to possess structural features enabling a mechanism of class discrimination to occur. The recent elucidation of the crystal structure of the ephrin-B1 ectodomain illustrates potential structural differences between two ephrin-Bs that could result in a greater affinity for one receptor than another (Nikolov et al., 2005).

1.3 Eph receptor/ephrin binding and activation

The membrane-bound nature of ephrins and Eph receptors means that their interaction requires intimate cell-cell contact. Activation of ephrins and Eph receptors leads to the multimerisation of both molecules into plasma membrane clusters (Davis et al., 1994). The degree of multimerisation required to achieve receptor activation varies between receptor-ligand pairs but it is thought that ephrins must be presented as higher order clusters to fully activate the Eph receptor response (Davis et al., 1994; Gale and Yancopoulos, 1997; Stein et al., 1998b).

Prior to cell-cell contact, unbound ephrins and Eph receptors are loosely pre-clustered into homodimers and found localised to cholesterol-rich lipid rafts (Bruckner et al., 1999). Interestingly, however, ephrin-B1 has recently been shown to be present as a monomer (Nikolov et al., 2005). Upon cell-cell contact, the ephrin ligands and Eph receptors bind with high affinity and 1:1 stoichiometry to form heterodimers (Himanen et al., 2001). The ligand molecules become re-orientated after receptor binding, and through complementary interaction surfaces dimer pairs join to form tetramers, consisting of two ephrins and two Eph receptors (Himanen and Nikolov, 2002; Himanen et al., 2001). The formation of this stable tetramer, where the carboxyl termini of both ephrin-B2 molecules are on the opposite side to the carboxy termini of the two EphB2 receptor molecules, allows signals to be generated in opposite directions at the cell-cell interface (Himanen et al., 2001).The tyrosine kinase domains of the paired receptor molecules *trans*-autophosphorylate each other and initiate forward signalling (Himanen et al., 2001). Interestingly, it has recently been demonstrated that new Eph receptors can be recruited into existing Eph-ephrin signalling clusters, resulting in a lateral association between Eph receptor

molecules on the same cell surface and this can occur independently of ephrin-binding. This could be responsible for an expansion of receptor clusters beyond the region of cell-cell contact (Wimmer-Kleikamp et al., 2004). Disruption of ephrin-ephrin homodimers and tetramer formation stimulates a conformational change in the transmembrane domain of the ephrin, altering the accessibility of tyrosine residues on the cytoplasmic tail (Himanen et al., 2001; Toth et al., 2001), known to become phosphorylated upon Eph receptor binding by cytoplasmic tyrosine kinases (Bruckner et al., 1997; Holland et al., 1996; Kalo et al., 2001). This initiates reverse signalling. A further conformational change in the cytoplasmic tail then occurs, changing it from a closed to an open conformation, leading to recruitment of various proteins involved in downstream signalling (Song, 2003). Ephrin-As do not possess a cytoplasmic domain but a region of ephrin-As has been postulated to be responsible for interaction with an ADAM metalloprotease, which has been shown to cleave the ephrin-A2 ectodomain from the cell surface after receptor binding providing a mechanism for cell-cell separation following high affinity binding of membrane bound Eph receptors and ephrins (Hattori et al., 2000).

1.4 Eph receptors and ephrins: Control of cell movements during morphogenesis

1.4.1 Development of the vasculature

During vertebrate development the vascular system is one of the first organ systems to function. Development of the mammalian vasculature is a highly specialised process requiring the organisation of blood vessels into a highly structured network. Initially this occurs through a process called vasculogenesis and is followed by angiogenesis (reviewed in Weinstein, 1999). Vasculogenesis involves the differentiation of angioblasts from mesoderm and the formation of primitive blood vessels at or near their site of origin. This results in the formation of the primary plexus, a basic undifferentiated structure lacking fine capillary networks, although a few major blood vessels are present such as the dorsal aorta (reviewed in Risau and Flamme, 1995). During angiogenesis, the primary vascular plexus undergoes remodelling to form the mature vascular network, involving the branching and

sprouting of new vessels together with the splitting and pruning of existing projections (reviewed in Risau, 1997). This is a complex process involving endothelial cell proliferation, chemotactic migration and functional maturation. A major aspect of angiogenesis is endothelial cell migration. Angiogenesis occurs throughout embryogenesis and also has roles in the adult during wound healing, the uterine cycle and placental growth as well as inflammatory responses and tumour neovascularisation.

1.4.1.1 Venous EphB4 and arterial ephrin-B2 expression

Until relatively recently it was thought that the difference in functional properties exhibited by mature arteries and veins arose as a reflection of vascular physiology. However, a number of groups have recently provided evidence that arterial and venous endothelial cells are molecularly distinct from the outset of angiogenesis, suggesting that the different functions exhibited by the two vessels are in part genetically determined. This distinction was revealed when arterial endothelial cells were found to endogenously express the transmembrane ligand ephrin-B2, and venous endothelial cells its cognate receptor EphB4 (Adams et al., 1999; Gerety et al., 1999; Wang et al., 1998).

EphB4/ephrin-B2 bi-directional signalling between arteries and veins was found to be necessary for remodelling the capillary network (Adams et al., 1999; Gerety et al., 1999; Wang et al., 1998). Mice lacking either ephrin-B2 or EphB4 die during embryogenesis around E10.5 with severe cardiovascular defects. Targeted disruption of the ephrin-B2 gene prevents the remodelling of both veins and arteries (Adams et al., 1999; Wang et al., 1998), yet the major vessels in the trunk form normally indicating vasculogenesis is not affected. A targeted mutation in EphB4 essentially phenocopies the defects seen in the ephrin-B2 mutant (Gerety et al., 1999), suggesting that EphB4 is the main functional partner of ephrin-B2 in the vasculature (Gerety et al., 1999), and that bi-directional signalling is required for correct angiogenic remodelling. In addition, the reciprocal expression pattern of ephrin-B2 on arteries and EphB4 on veins, and the maintenance of arterial/venous identity into fine capillary beds, where arteries and veins make contact (Wang et al., 1998),

suggests a role for EphB4/ephrin-B2 signalling in defining arterial/venous boundaries possibly by preventing cell mixing (Adams et al., 1999; Gerety et al., 1999; Wang et al., 1998). In support of this, endothelial cell lines expressing EphB4 or ephrin-B2 will segregate when mixed (Fuller et al., 2003).

In order to elucidate the importance of reverse signalling in maintaining the arterial/venous boundary, the role of the ephrin-B2 cytoplasmic domain *in vivo* was investigated. Knock-in mutant mice were generated in which endogenous ephrin-B2 was replaced with truncated ephrin-B2 lacking the cytoplasmic domain (ephrinB2$^{\Delta C}$) (Adams et al., 2001). Truncated ephrin-B2 is unable to exert reverse signals via its cytoplasmic domain, therefore is restricted to the induction of forward signalling by Eph receptors on adjacent cells. Homozygous ephrinB2$^{\Delta C/\Delta C}$ mice exhibited defects in angiogenic remodelling reminiscent of the ephrin-B2 knock-out mice (Adams et al., 2001). Truncated ephrin-B2 was able to rescue the neural crest cell defects seen in ephrin-B2 knockout mice confirming that forward signalling through the receptor was functioning (Adams et al., 2001). This finding indicated that the ephrin-B2 cytoplasmic domain, and reverse signalling generated through ephrin-B2, was required for correct vascular morphogenesis. However, this observation was challenged in a later study where the cytoplasmic domain was replaced by ß-galactosidase, which did not result in angiogenic defects (Cowan et al., 2004). This indicated the C-terminal domain of ephrin-B2 was not required for early vascular development. The different outcomes observed could be due to the type of mutations used leading to altered protein localisation which may prevent interaction with EphB receptors, or could affect their ability to cluster and function. Cowan and colleagues demonstrated that a truncated ephrin-B2 became trapped in the trans-golgi network failing to reach the plasma membrane, in effect acting as a protein null (Cowan et al., 2004). This could have been the case in the earlier study. However, despite not playing a role in early vascular development, the cytoplasmic domain of ephrin-B2, and therefore reverse signalling, was found to be necessary for the correct formation of the cardiac valve (Cowan et al., 2004).

In embryos EphB4 expression is restricted to the cardiovascular system (Gerety et al., 1999). However, delayed expression (onset E12.5) of ephrin-B2 has been shown in mesenchymal cells, pericytes and vascular smooth muscle cells (Adams et al., 1999; Gale et al., 2001; Shin et al., 2001a; Wang et al., 1998). Since expression of ephrin-B2 is not restricted solely to the vasculature, previous studies could not definitively distinguish between a vascular or mesenchymal requirement. Knocking out ephrin-B2 specifically in the vasculature demonstrated the absolute requirement for ephrin-B2 in order for correct angiogenic remodelling of both arteries and veins to occur (Gerety and Anderson, 2002). Mesenchymal ephrin-B2 alone is not sufficient to support angiogenesis in the absence of vascular ephrin-B2, demonstrated by indistinguishable cardiac defects exhibited in the vascular ephrin-B2 knockout and the conventional knockout (Gerety and Anderson, 2002; Wang et al., 1998).

1.4.1.2 Other Eph/ephrin interactions in the developing vasculature

Eph/ephrin expression in the vascular system is not simply restricted to EphB4 and ephrin-B2, other receptors and ligands are also expressed although their role in angiogenesis is less clear. Ephrin-B1 is co-expressed with ephrin-B2 in arteries, together with EphB3 on aortic arches. Ephrin-B1 and EphB3 are co-expressed with EphB4 in veins (Adams et al., 1999) and expression of EphB2, EphB3 and EphB4 together with ephrin-B1 and ephrin-B2 was found in yolk sacs (Adams et al., 1999). In addition EphB2 and ephrin-B2 are expressed in mesenchymal cells adjacent to endothelial cells (Adams et al., 1999). This expression analysis suggests complex cell-cell interactions, via ephrins and Eph receptors, between endothelial cells throughout the vasculature and between endothelial and mesenchymal cells.

Although ephrin-B1 is co-expressed with ephrin-B2 in arteries, it is unable to rescue the vascular defects resulting from the ephrin-B2 mutation, suggesting these ligands have distinct functions in the developing vasculature (Adams et al., 1999). Neither EphB2 or EphB3 knockout mice show any vascular defects (Adams et al., 1999). However, EphB2/EphB3 double mutant embryos display a 30% penetrant phenotype that resembles the ephrin-B2 mutant, although less severe (Adams et al., 1999).

Prominent expression of EphB2 and ephrin-B2 in mesenchymal cells adjacent to endothelial cells also implicates ephrins and Eph receptors in interactions between endothelial and mesenchymal cells (Adams et al., 1999).

In mice, intersomitic vessels that form at somite boundaries express EphB3 and EphB4 receptors (EphB4 in *Xenopus*) at a time when ephrin-B2 is expressed in the caudal half of the somites (ephrin-B1 and ephrin-B2 in *Xenopus*) (Adams et al., 1999; Helbling et al., 1999). This close contact between ephrin-B expressing cells of the dermomyotome and EphB expressing endothelial cells suggested a functional interaction of intersomitic vessels and the connecting capillary network. Ephrin-B2 null mutants showed aberrant organisation and branching of intersomitic vessels, compared to their wild-type counterparts (Adams et al., 1999). In addition, constitutive, ubiquitous overexpression of ephrin-B2 results in an abnormal segmental arrangement of intersomitic vessels (Oike et al., 2002). However, overexpression of ephrin-B2 solely in vascular endothelial cells does not, suggesting non-endothelial ephrin-B2 expression alters the migration of EphB expressing endothelial cells into the intersomitic region where ephrin-B2 expression is normally absent (Oike et al., 2002). In *Xenopus*, expression of dominant negative EphB4 lacking the intracellular tyrosine kinase domain or overexpression of ephrin-B2 resulted in abnormal projection of intersomitic veins into adjacent somites. Ephrin-B reverse signalling was not required. Therefore, forward signalling through EphB4 is sufficient for directional growth of intersomitic veins (Helbling et al., 2000). Abnormal dorsal branches were also observed in EphB2/EphB3 double mutants (Adams et al., 1999). A function of the interaction between somitic ephrin-B2 and endothelial EphB receptors may be to suppress sprouts which are therefore seen in the mutant (Adams et al., 1999).

Various EphA receptors and ephrin-A ligands are expressed in the developing vasculature of the mouse (Flenniken et al., 1996; McBride and Ruiz, 1998). However, no vascular defects have been detected as a result of knocking out EphA receptors or ephrin-A ligands (Dottori et al., 1998; Feldheim et al., 2000; Frisen et al., 1998). It remains to be determined whether EphA receptors and ephrin-As have a function in vascular development.

1.4.1.3 Eph/ephrin regulation of cell migration and angiogenic sprouting

During angiogenesis, migration of endothelial cells to their correct targets is essential. Numerous *in vitro* studies have investigated the effects of Eph receptors and ephrin ligands on endothelial cell migration and sprouting. However, conflicting results have been obtained from such experiments. Some studies describe Eph receptors and/or ephrins as positive regulators of migration and sprouting while others describe the opposite result. These discrepancies could be due to the many different cell types involved, which express different Eph receptors and ephrins, and the promiscuous binding capabilities of Eph receptors and ephrins. In addition the experimental conditions are varied. Further work is needed to clarify the situation.

Stimulation of EphB receptor expressing endothelial cells with ephrin-B2-Fc or contact with ephrin-B2 expressing cells can result in increased cell migration (Maekawa et al., 2003; Nagashima et al., 2002; Sawai et al., 2003; Steinle et al., 2002), proliferation (Steinle et al., 2002), and sprouting (Adams et al., 1999; Stein et al., 1998b; Zhang et al., 2001). For example, stimulation of adrenal cortex-derived microvascular endothelial (ACE) cells expressing EphB receptors, with soluble ephrin-B1-Fc or ephrin-B2-Fc induced significant sprouting activity (Adams et al., 1999). Interestingly, unclustered ephrin-B1 was sufficient to induce sprouting where as ephrin-B2 had to be pre-clustered in order to show the same response (Adams et al., 1999). In contrast, other studies have demonstrated that EphB forward signalling results in a decrease in cell migration, proliferation and sprouting (Fuller et al., 2003; Hamada et al., 2003; Kim et al., 2002; Sturz et al., 2004).

EphB4-Fc stimulation of ephrin-B2, and activation of ephrin-B2 reverse signalling, has also been shown to increase cell migration (Fuller et al., 2003; Hamada et al., 2003; Huynh-Do et al., 2002; Steinle et al., 2003), sprouting (Adams et al., 2001) and proliferation (Steinle et al., 2003). Both ephrin-B1 and ephrin-B2 display an *in vitro* capillary sprout-inducing ability (Adams et al., 1999). In addition, soluble EphB3-Fc and EphB4-Fc (Adams et al., 2001; Palmer et al., 2002), but not EphB2-Fc (Adams et al., 2001) or EphB1-Fc (Adams et al., 1999) were able to induce

sprouting, suggesting there is a degree of specificity required, perhaps mediated through receptor-ligand binding affinities (Adams et al., 2001). However the opposite effects have also been documented. Growing ephrin-B2 expressing cells on EphB4 expressing cells inhibits angiogenic sprouting (Zhang et al., 2001).

In vivo evidence also exists for EphB/ephrin-B signalling influencing cell migration and sprouting. Ephrin-B2 deficient embryos show defective vascularisation of the nervous system, which normally occurs via angiogenic sprouting from the perineural vascular plexus. Therefore, ephrin-B2 may induce capillary sprouting *in vivo* (Adams et al., 1999). Furthermore, ephrin-B2-Fc stimulation of EphB expressing HUVECs promotes angiogenesis *in vivo* during a mouse corneal neovascularisation assay (Maekawa et al., 2003).

EphA/ephrin-A signalling has also been shown to play a role in endothelial cell migration. Ephrin-A1 promotes angiogenesis *in vivo* as well as endothelial cell migration *in vitro* (Brantley et al., 2002; Daniel et al., 1996; Pandey et al., 1995). EphA activation has been shown to be required for VEGF-mediated endothelial cell sprouting in an *in vitro* capillary sprouting assay (Cheng et al., 2002b). In addition, EphA2 receptor signalling is required for capillary sprouting in HUVECs (Ogawa et al., 2000). Interestingly, it has recently been reported that EphA2 activation triggers an inhibition of endothelial cell migration (Ojima et al., 2006).

Genetic studies have unambiguously demonstrated a requirement for Eph/ephrin signalling during angiogenesis. However *in vitro* data, dissecting the cellular responses to Eph receptor and ephrin activation, presents a conflicting picture of both repulsive and attractive outcomes of Eph and ephrin activation.

1.4.1.4 Role of Eph/ephrin signalling during adult angiogenesis

Arterial ephrin-B2 expression and venous EphB4 expression is maintained throughout later embryogenesis and in the adult, with expression extending to the microvasculature in a variety of tissues (Gale et al., 2001; Shin et al., 2001a). Expression of ephrin-B2 is also found in the smooth muscle cells surrounding the

vessels (Gale et al., 2001; Shin et al., 2001a). In the adult, ephrin-B2 expression in newly forming blood vessels was observed both in normal and pathological settings, such as in the female reproductive system, during wound healing and in tumours (see 1.6.2 for Eph/ephrin involvement in cancer).

One example of EphB/ephrin-B signalling in physiological angiogenesis in the adult is during follicular and corpus luteum formation in the adult ovary (Egawa et al., 2003; Gale et al., 2001). High levels of ephrin-B2 expression have been described during follicular maturation and differentiation of the ovum, to form a highly vascularised corpus luteum (Gale et al., 2001). EphB1, B2 and B4 together with ephrin-B1 and B2 are expressed in human corpora lutea of the early luteal phase. A rapid increase in ephrin-B1 expression was observed on human luteinizing granulosa cells after ovulation, and ephrin-B1 was able to bind EphB2-Fc suggesting ephrin-B1 expressing cells may directly interact with Eph bearing cells *in vivo* during corpus luteum formation (Egawa et al., 2003). EphB4 and ephrin-B2 have also been implicated in the hormone-dependent morphogenesis of the mammary gland. Expression of EphB4 and ephrin-B2 are induced at the onset of puberty. EphB4 expression is localised to the myoepithelial cells of ducts and alveoli, and ephrin-B2 to the luminal epithelial cells. Expression is oestrogen induced and levels are at their highest during the proliferative stages, associating them with growth and/or tissue remodelling (Nikolova et al., 1998).

Eph/ephrin signalling is also involved in pathological angiogenesis. Neovascularisation in the retina is a critical component of conditions that can result in blindness, such as age-related macular degeneration, diabetes mellitus and retinopathy of prematurity (ROP) (Lee et al., 1998), and Eph receptors and ephrins have recently been identified as mediators of retinal neovascularisation. Ephrin-B2, EphB2 and EphB3 are expressed in the endothelial cells of fibroproliferative membranes of patients with proliferative diabetic retinopathy (PDR) and ROP (Umeda et al., 2004) and ephrin-A1 stimulates corneal angiogenesis *in vivo* (Pandey et al., 1995). In support of *in vitro* data demonstrating the presence of ephrin-B2 and EphB4 expression in retinal endothelial cells (Steinle et al., 2003) and regulation of endothelial cell migration via both forward and reverse signalling (Steinle et al., 2003; Steinle et al., 2002), soluble ephrin-B2 and EphB4 have been shown to reduce

retinal neovascularisation in a murine model of proliferative neuropathy (Zamora et al., 2005), and recently, ephrin-A1 has been shown to do the same (Ojima et al., 2006). Inhibition of EphA receptors by blocking the interaction between ephrin-A ligands and endogenous EphA receptors using soluble EphA2-Fc treatment has been shown to inhibit the severity of abnormal retinal neovascularisation in a rat model of ROP, without affecting normal vessel development. In addition, soluble EphA2-Fc inhibits ephrin-A1 induced corneal angiogenesis *in vivo* (Chen et al., 2005; Cheng et al., 2002b). Soluble EphA2-Fc treatment inhibited retinal endothelial cell migration and the formation of tube-like structures in response to either ephrin-A1 or VEGF and the results from EphA2 deficient mice would suggest that the specific receptor involved is EphA2 (Chen et al., 2005).

1.4.1.5 Cross-talk with other mediators of vascular development

Three main families of receptor tyrosine kinases are known to be mediators of angiogenesis: vascular endothelial growth factor receptor (VEGFR), Tie and Eph receptors. They act with their ligands: vascular endothelial growth factors (VEGFs), Angiopoietins and ephrins respectively, to pattern the vasculature (reviewed in Yancopoulos et al., 2000).

VEGF has been found to co-localise with ephrin-A1 and EphA2 in tumour sections (Brantley et al., 2002), and EphA2-Fc can inhibit VEGF-induced endothelial cell migration suggesting cooperation between these signalling pathways (Brantley et al., 2002). Furthermore, VEGF-induced angiogenesis *in vivo* specifically requires EphA receptor activation (Cheng et al., 2002b). Cross-talk is not simply restricted to VEGF, the angiogenic phenotype in the head and yolk sac of the ephrin and Eph knockout mice appears similar to that of mutations in mice where the receptor Tie-2 and its ligand angiopoietin-1 have been knocked out (Sato et al., 1995; Suri et al., 1996), and Tie2 has been shown to phosphorylate the cytoplasmic domain of ephrin-B1 (Adams et al., 1999).

1.4.1.6 Ephrin-B signalling during lymphangiogenesis

Ephrin-B2 signalling has recently been shown to play a role not only in the development of blood vessels but also in the lymphatic system (Makinen et al., 2005). The major roles of the lymphatic system are to maintain tissue fluid balance, provide a route for fat absorption in the gut and immune surveillance. Lymphatic vessels appear to originate from a subset of venous endothelial cells and like the blood vasculature the lymphatic system undergoes remodelling. However, the development of the lymphatic vasculature is less understood. Makinen and colleagues, demonstrated a requirement *in vivo* for the PDZ binding motif at the C-terminal end of ephrin-B2 in order for correct lymphangiogenic remodelling (Makinen et al., 2005). Two knock-in mice were generated. Ephrin-$B2^{\Delta V/\Delta V}$ mice expressed ephrin-B2 lacking the C-terminal valine in the PDZ binding site and ephrin-$B2^{5F/5F}$ mice expressed ephrin-B2 with 5 conserved tyrosine residues mutated to phenylalanine. Homozygous ephrin-$B2^{5F/5F}$ mutant mice survived the requirement for ephrin-B2 in the vascular system, however, homozygous ephrin-$B2^{\Delta V/\Delta V}$ mutant mice exhibited major lymphatic defects (Makinen et al., 2005). Interestingly, ephrin-B2 reverse signalling was also implicated in the elongation and guidance of sprouting lymphatic endothelial cells. Wild-type mice showed filopodial extensions at sites of initiation of lymphatic endothelial cell sprouting. However, sprouting was disrupted in the ephrin-B2 PDZ mutant mice resulting in blunt ended protrusions (Makinen et al., 2005). Interaction with PDZ proteins but not tyrosine phosphorylation was found to be required for lymphatic remodelling.

1.4.2 Regulation of cell migrations and tissue segmentation

During embryogenesis many invertebrates and all vertebrate embryos undergo the basic process of segmentation. In vertebrates there are two clearly segmented regions of the body axis, the paraxial mesoderm and the hindbrain region of the neural plate. Eph receptors and ephrins exhibit multiple functions in tissue patterning and boundary formation during segmentation and regulate cell migrations during these events. Expression of Eph receptors and ephrins can be reciprocal or overlapping and interactions can lead to both attractive and repulsive responses.

1.4.2.1 Vertebrate hindbrain

Development of the vertebrate hindbrain occurs by the formation of a series of distinct segments via subdivision of the neural epithelium into at least 5 compartments called rhombomeres, numbered r2 through to r6 (Fraser et al., 1990). Rhombomere formation depends on the expression of genes encoding transcription factors such as the Hox group 1-4 protein, Krox-20 and Valentino/Kreisler/MafB whose expression is restricted to specific segements (Cooke and Moens, 2002). As each rhombomere is formed, cell mixing across segment boundaries is restricted (Fraser et al., 1990).

Donor-to-host transplantation, where quail rhombomeres were grafted into a chick host, demonstrated that negligible cell mixing occurred when r4 was positioned adjacent to r3. However, no boundary was formed when r3 was placed next to r5 or r3. Interestingly, cell mixing was more extensive when identical rhombomeres were grafted next to each other (Guthrie et al., 1993). Taking this further, Guthrie and colleagues demonstrated that upon grafting of fluorescently labelled chick rhombomere tissue into various rhombomeres of chick hosts, sorting would occur when cells were transplanted into an adjacent segment. However, cells grafted into the same position or two segments distant would not undergo sorting, cells would disperse throughout the population. For example, cells from r3 would mix when grafted into r3 or r5 of the host (Guthrie et al., 1993).

Eph receptors and ephrins are expressed segmentally in the developing hindbrain. EphB2, EphB3 and EphA4 are expressed in the odd numbered rhombomeres r3 and r5 (Becker et al., 1994; Henkemeyer et al., 1994; Nieto et al., 1992). Ephrin-B1, -B2 and -B3 are expressed in the even numbered rhombomeres r2, r4 and r6 (Bergemann et al., 1995; Flenniken et al., 1996; Gale et al., 1996a). Eph receptor and ephrins are therefore in contact at rhombomere boundaries, ideally located for restriction of cell intermingling. The segmental expression of Eph receptors and ephrins in this way is likely to be under the control of transcriptional regulators, which also have a rhombomere specific expression. EphA4 has been shown to be a direct transcriptional target of Krox-20, and Valentino is required to establish

complementary domains of EphA4 and ephrin-B2 in the caudal hindbrain of zebrafish (Cooke and Moens, 2002).

Experiments in zebrafish suggested that Eph receptor kinases were indeed required for the segmental restriction of cells (Xu et al., 1995; Xu et al., 1999). Expression of truncated EphA4 receptor resulted in disruption of hindbrain segmentation. Cells with r3/r5 identity were found within even numbered rhombomeres (Xu et al., 1995). Further evidence was obtained through mosaic analyses of exogenous ephrin-B2 expression in zebrafish embryos. Control RNA injected at the 8-cell stage illustrated a scattered distribution of expressing cells throughout the hindbrain. Ephrin-B2 expression resulted in sorting of ephrin-B2 expressing cells to the boundaries of r3/r5, while in rhombomeres r2/r4/r6 many expressing cells were located centrally. Cells expressing truncated ephrin-B2 exhibited a similar expression pattern indicating that mosaic activation of Eph receptors by ephrin-B2 is sufficient for cell sorting (Xu et al., 1999). However, cells expressing truncated EphA4 or truncated EphB2 were found to sort to the boundaries of r2/r4/r6 and centrally within r3/r5. This result suggests that signalling via ephrin-Bs may also occur at rhombomere boundaries to restrict cell intermingling (Xu et al., 1999).

In order to analyse further whether unidirectional or bidirectional signalling between complementary expression patterns of Eph receptors and ephrins restrict intermingling, an assay was developed by Mellitzer and colleagues using zebrafish animal caps. Mixing two cell populations from zebrafish animal caps, each labelled with a fluorescent tracer, resulted in extensive cell intermingling (Mellitzer et al., 1999). If cells were co-injected with RNA so that ephrin-B2 was expressed in one cell population and EphB2/EphA4 in another cell intermingling was restricted. Sorting of cells to different domains in this way was shown to be dependent on bi-directional signalling. Allowing only unidirectional signalling by injection of truncated versions of either ephrin-B2 or EphB2, lacking the intracellular domain, adjacent to the full-length binding partner, failed to restrict cell intermingling (Mellitzer et al., 1999). Transfer of the dye Lucifer Yellow between cells also assessed gap junctional communication. Although bidirectional signalling did disrupt gap junctional communication, unidirectional signalling through Eph receptors was shown to be sufficient for this to occur (Mellitzer et al., 1999) and gap junctional

communication is a feature of rhombomere boundaries *in vivo*. Whereas unidirectional signalling was sufficient to restrict cell mixing in the context of the hindbrain this was not the case in this "fishball" assay. Perhaps additional mechanisms compensated for the lack of bidirectional signalling in the hindbrain situation, allowing cell sorting to occur (Cooke and Moens, 2002).

Recently, Cooke and colleagues eliminated EphA4 and ephrin-B2a expression in zebrafish using antisense morpholinos (EfnB2, the gene encoding ephrin-B2, has two orthologs in zebrafsh ephrinB2a and ephrin-B2b). Loss of EphA4 function results in disruption of rhombomere boundaries consistent with a role for EphA4 signalling. However, in mosaic embryos, EphA4 loss of function cells sorted from wild type EphA4 expressing cells, suggesting that EphA4 promotes cell adhesion within the rhombomeres in which it is expressed. This led to the hypothesis that two mechanisms are operating in parallel, the well-known EphA4-dependent repulsion together with EphA4-dependent adhesion within rhombomeres (Cooke et al., 2005).

1.4.2.2 Somitogenesis

The process of somitogenesis is the formation of somites, which form progressively along the antero-posterior axis from the pre-somitic mesoderm, by the aggregation of mesenchymal cells and their transformation into epithelial balls (somites), separated by intersomitic furrows. The genetic factors that underlie the early patterning of the pre-somitic mesoderm are well known (Maroto and Pourquie, 2001). These repeating units are the precursors to the vertebral column and each somite is subdivided into a rostral (anterior) and caudal (posterior) domain, the morphogenetic capabilities of which differ (Goldstein and Kalcheim, 1992). It has been demonstrated by grafting experiments in the chick that interfaces between anterior and posterior regions of the somite are required for the maintenance of somite boundaries (Stern and Keynes, 1987). Like rhombomeres, somites display alternating patterns of Eph receptor and ephrin expression. However, Eph receptor and ephrin expression domains are restricted to the anterior and posterior halves of a single somite. EphB receptors are expressed in the anterior half of somites and ephrin-B ligands in the posterior half (Bergemann et al., 1995; Flenniken et al., 1996; Holder

and Klein, 1999). In zebrafish, interference with Eph receptor/ephrin signalling by expressing dominant negative EphA4 or soluble ephrins results in abnormal boundary formation (Durbin et al., 1998), indicating a role for Eph receptor/ephrin signalling in restricting intermingling between the anterior and posterior halves. It has also been shown that re-expressing EphA4 and ephrin-B ligands in alternating patterns can rescue segmentation should the somites become fused (Barrios et al., 2003; Durbin et al., 1998) and Eph/ephrin signalling is essential for a correct mesenchymal to epithelial transition (Barrios et al., 2003).

1.4.2.3 Trunk neural crest cell migration

Neural crest cells arise by delamination of cells from the dorsolateral edge of the neural epithelium and migrate along specific pathways to their destinations where they differentiate into an array of cell types from neurons to pigment cells. Trunk neural crest cells take two distinct migratory pathways and have different developmental fates. Initially the majority of neural crest cells migrate ventrally through the anterior half of the somites avoiding the posterior half (Bronner-Fraser, 1993), and differentiate into dorsal root and sympathetic ganglia, schwann cells and a number of other derivatives (Goldstein and Kalcheim, 1991). Cells migrating from the neural tube 12-18 hours later invade the dorsolateral pathway between the dermomyotome and the overlying ectoderm (Erickson et al., 1992; Serbedzija et al., 1989), where they differentiate into melanocytes.

The segmental expression of ephrins in the somites is thought to guide early neural crest cell migration. Trunk neural crest cells, which express EphB receptors, will migrate from the neural tube avoiding the posterior half of the somites, which express ephrin-B ligands. Different B receptors and ligands can perform the task in different species. In the chick, crest cells and cells in the anterior half of the somite express EphB3 and the posterior half of the somites express ephrin-B1 (Krull et al., 1997). In rodents EphB2 and ephrin-B2 are the receptor and ligand involved (Wang and Anderson, 1997). This was demonstrated by the repulsive response seen to ephrin-B proteins, which repelled trunk neural crest cells during *in vitro* stripe assays (Krull et al., 1997; Wang and Anderson, 1997). Stripes of ephrin-B1 or ephrin-B2

were laid down on a matrix and the behaviour of cells on the stripes analysed. It was demonstrated that neural crest cells migrating out of a dissected neural tube avoid ephrin-B stripes, contact with which causes the collapse and retraction of cell processes (Krull et al., 1997; Wang and Anderson, 1997). Blocking experiments using soluble ephrin-B1 have also demonstrated that EphB-ephrin-B signalling is required to prevent neural crest cells entering the posterior half of the somite in rodent and avian explants (Koblar et al., 2000; Krull et al., 1997) and time-lapse analysis revealed that cells become disorientated and even moved backwards into the neural tube (Krull et al., 1997). Inhibition of neural crest cell migration was also demonstrated using a chemotaxis assay (Wang and Anderson, 1997). However, a null mutation in ephrin-B2 does not affect neural crest cell migration. This could be due to the continued presence of other guidance cues, which can compensate for the mutation (Wang and Anderson, 1997).

Neural crest cells migrating at later stages differentiate to form melanoblasts that migrate along a dorsolateral route. The restriction to a ventral route is in part regulated by ephrin-B ligands expressed in the posterior half of the somites (Krull et al., 1997; Wang and Anderson, 1997) and in the dermomyotome (Santiago and Erickson, 2002) acting as a repulsive cue to early migrating cells so they are unable to enter the dorsolateral pathway. However, dorsolateral expression of ephrin-B ligands is not downregulated at the time of melanoblast migration (Koblar et al., 2000; Santiago and Erickson, 2002). A switch occurs where ephrin-B ligands now appear to act as a positive cue required for the migration of these cells along the dorsolateral pathway (Santiago and Erickson, 2002). The work of Santiago and Erickson puts forward three lines of evidence to support this switch to an attractive role. Firstly, melanoblasts express several EphB receptors and blocking receptor activation by addition of soluble ligand to trunk explants resulted in cells no longer migrating via a dorsolateral route. Secondly, a chemotaxis assay demonstrated that soluble ephrin-B1 inhibits the migration of early neural crest cells, but promotes the migration of those leaving the neural tube later. Finally, engagement of Eph receptors by substrate bound ephrin-B ligands promotes melanoblast attachment to fibronectin. Early neural crest cells lose their actin stress fibres and round up in response to binding ephrin-B, whereas late neural crest cells form microspikes

containing filamentous actin (Santiago and Erickson, 2002). It is currently not known how the switch in cell responses to EphB activation is achieved.

1.4.2.4 Cranial neural crest cell migration

Segmental migration of neural crest also occurs from rhombomeres to specific branchial arches, where they differentiate to form distinct patterns of cartilage and skeletal structures (Lumsden and Guthrie, 1991; Serbedzija et al., 1992). In *Xenopus*, mouse and chick, the neural crest is divided into three streams corresponding to specific rhombomeres (r) and branchial arches. Neural crest cells migrate from r2, r4 and r6 to the first, second and third plus fourth branchial arches respectively (Fig. 1.6). The third stream splits into two parts during migration in order to enter both the third and fourth arch (Fig. 1.6) (Sadaghiani and Thiebaud, 1987; Sechrist et al., 1993; Serbedzija et al., 1992).

Figure 1.6 Cranial neural crest cell migration

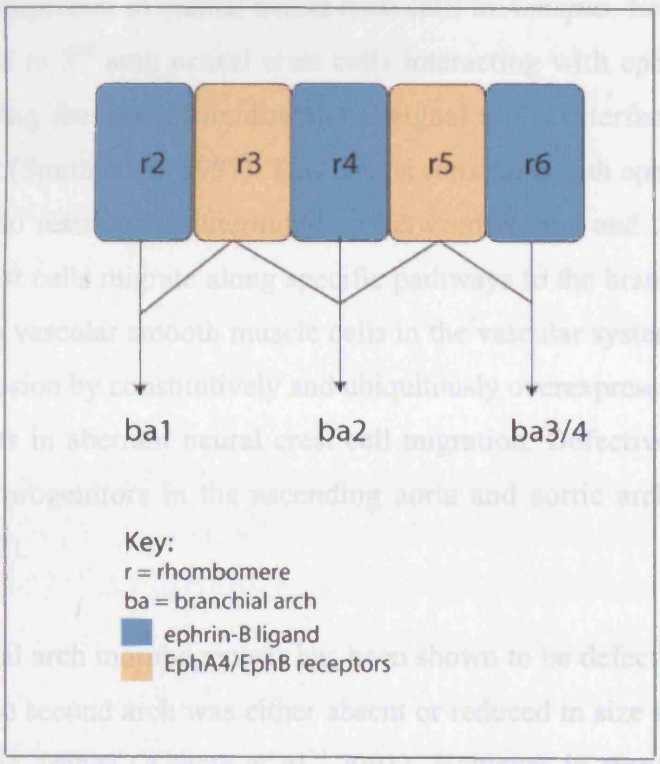


Figure legend 1.6 - Cranial neural crest cells migrate in separate streams from specific rhombomeres. Rhombomere 2 gives rise to cells that populate branchial arch 1, rhombomere 4 gives rise to cells that populate branchial arch 2 and neural crest cells from rhombomere 6 migrate to branchial arches 3 and 4. Neural crest cells from rhombomeres 3 and 5 migrate with the adjscent streams. The complementary expression of ephrin-B2 and EphA4/EphB1 in specific rhombomeres is thought to be involved in the targeted migration of cranial neural crest cells.

In *Xenopus* the complementary expression of ephrin-B2 in second arch neural crest and mesoderm and of EphA4 plus EphB1 in third arch neural crest and mesoderm has been implicated in the targeted migration of cells (Smith et al., 1997). Expression of truncated EphA4 or EphB1 resulted in migration of 3rd arch neural crest cells into the normally restricted adjacent 2nd arch territory, while the migration of 2nd arch neural crest cells remained unaffected (Smith et al., 1997). Overexpression of a kinase dead form of EphA2 also results in cranial neural crest defects where the third and fourth streams do not separate properly. The defects are rescued upon expression of full-length EphA2 (Helbling et al., 1998).This suggests a functional role for Eph receptors in the migration of cranial neural crest cells in *Xenopus*. Ectopic expression of ephrin-B2 led to 3rd arch neural crest cells interacting with ephrin-B2 from all directions masking the normal unidirectional signal at the interface of 2nd and 3rd arch neural crest (Smith et al., 1997). This data is consistent with ephrin-B2 acting as a repulsive cue to restrict cell intermingling between second and third arch neural crest. Neural crest cells migrate along specific pathways to the branchial arches and differentiate into vascular smooth muscle cells in the vascular system. Disruption of ephrin-B2 expression by constitutively and ubiquitously overexpressing ephrin-B2 in the mouse results in aberrant neural crest cell migration. Defective recruitment of smooth muscle progenitors in the ascending aorta and aortic arch were detected (Oike et al., 2002).

In mice, branchial arch morphogenesis has been shown to be defective in ephrin-B2 null mutants. The second arch was either absent or reduced in size and there was an absence of aortic arches (Adams et al., 2001). However in truncated ephrin-B2 mutants where the cytoplasmic domain of ephrin-B2 was missing, no such defects

occurred. Therefore, truncated ephrin-B2 rescued the branchial and aortic defects seen in ephrin-B2 null mutants. The guidance of neural crest cells was also disrupted in ephrin-B2 null mutants. The cells did not migrate in a well-defined stream and entered areas they were normally restricted from. Once again, however, truncated ephrin-B2 could largely restore the normal cell migration pattern (Adams et al., 2001). This work demonstrates a requirement for the extracellular domain of ephrin-B2, but not the cytoplasmic domain, for cranial neural crest cell migration.

However, a requirement for ephrin-B1 forward and reverse signalling for correct branchial neural crest cell migration has recently been demonstrated (Davy et al., 2004). Ephrin-B1 mutant mice exhibit a cleft palate and disordered migration that is also seen when ephrin-B1 is conditionally knocked out in neural crest cells or when ephrin-B1 with a defect in the cytoplasmic domain is expressed indicating reverse signalling through ephrin-B1 is playing a role (Davy et al., 2004).

1.5 Axon guidance

During development, neurons project axons at great distances to reach their final targets in order to establish a functional nervous system. At the leading edge of a growing axon is a structure known as the growth cone, which is composed of lamellipodia, containing cross-linked networks of actin filaments, and filopodia, which are tensile structures composed of bundled F-actin that probe the extracellular environment. It is the response to extracellular guidance cues detected by the growth cone that determines whether axons terminate at their appropriate location, regulating the speed and direction of axon outgrowth.

There are a large number of both positive and negative regulators of axon guidance, and many of these guidance cues are bi-functional; able to act as both repellents and attractants. The main families known to be involved so far include the netrins, slits, semaphorins and ephrins, together with their cell-surface receptors, which have been shown to govern cytoskeletal dynamics in axonal growth cones. Although Eph/ephrin signalling has been shown to function widely throughout the nervous system, such as in the guidance of commissural axons (Imondi and Kaprielian, 2001;

Imondi et al., 2000), and guidance of corticospinal tract axons (Dottori et al., 1998), one of the most widely used models to study axon guidance is the projection of retinal axons to the midbrain. This section will focus on the involvement of Eph receptors and ephrins specifically, in the guidance of axons to their appropriate targets for the formation of topographic maps.

1.5.1 Formation of the retinotectal map

A topographic map is a projection from one set of neurons to another with the receiving cells mirroring the relationships of the projecting cells. In the retinotectal map, axons from the temporal retina project to the optic tectum (OT) in the chick, amphibians and fish, and to the superior colliculus (SC) in mammals. Mapping occurs along two axes: the temporal-nasal (TN) axis of the retina along the anterior-posterior (AP) axis of the OT/SC, and the dorsal-ventral (DV) axis of the retina along the lateral-medial (LM) axis of the OT/SC. Therefore, axons from increasingly nasal parts of the retina project to increasingly posterior parts of the tectum (Fig. 1.7), and a similar projection of axons occurs along the dorsal-ventral axis, with axons from the dorsal retina migrating to the ventral retina and vice versa (Fig. 1.8) (reviewed in Thanos and Mey, 2001). In mice, the primary axons overshoot their ultimate target position (Roskies and O'Leary, 1994) and interstitial branches are sent out at the anterior-posterior level corresponding to the final termination position. These branches then extend laterally and medially towards the termination zone (TZ) appropriate to the NT and DV retinal origin, where they finally arborise. Eph/ephrin signalling has been found to be crucial for all steps throughout this process.

1.5.1.1 Patterning the anterior-posterior axis

The Eph/ephrin family of receptors and ligands meet all the criteria for a topographic guidance molecule: they are expressed in a graded manner in the retina or OT/SC; Retinal ganglion cell (RGC) axons from different parts of the retina exhibit different responses to them; they affect RGC mapping *in vivo*. A gradient of ephrin-A expression is found in the tectum, which guides retinal axons to form the AP axis (Fig.1.7). Ephrin-A2 (Cheng et al., 1995) and ephrin-A5 (Drescher et al., 1995) are

expressed in high posterior to low anterior gradients in the tectum (Cheng et al., 1995; Connor et al., 1998; Drescher et al., 1995). Ephrin-A2 is expressed in a fairly shallow gradient across the entire tectum, whereas ephrin-A5 is expressed along a steeper gradient, but is restricted to the posterior half (Monschau et al., 1997). Complementary to ephrin-A2 and ephrin-A5 expression in the tectum, EphA3 (chick) or EphA5/EphA6 (mouse) are expressed in an increasingly nasal to temporal gradient in the retina (Cheng et al., 1995; Connor et al., 1998; Feldheim et al., 1998). Temporal axons express high levels of EphA3 and are sensitive to activation by ephrin, such that repulsion occurs in the anterior tectum, where there are low levels of ephrin expression. In contrast, nasal axons, which express low levels of EphA3, are less sensitive and can therefore migrate further into the posterior tectum, where there are high levels of ephrin expression. Repulsion of EphA expressing retinal ganglion cells in this way allows establishment of the AP axis.

Figure 1.7 Gradients of Eph receptor and ephrin expression along the AP axis in the chick

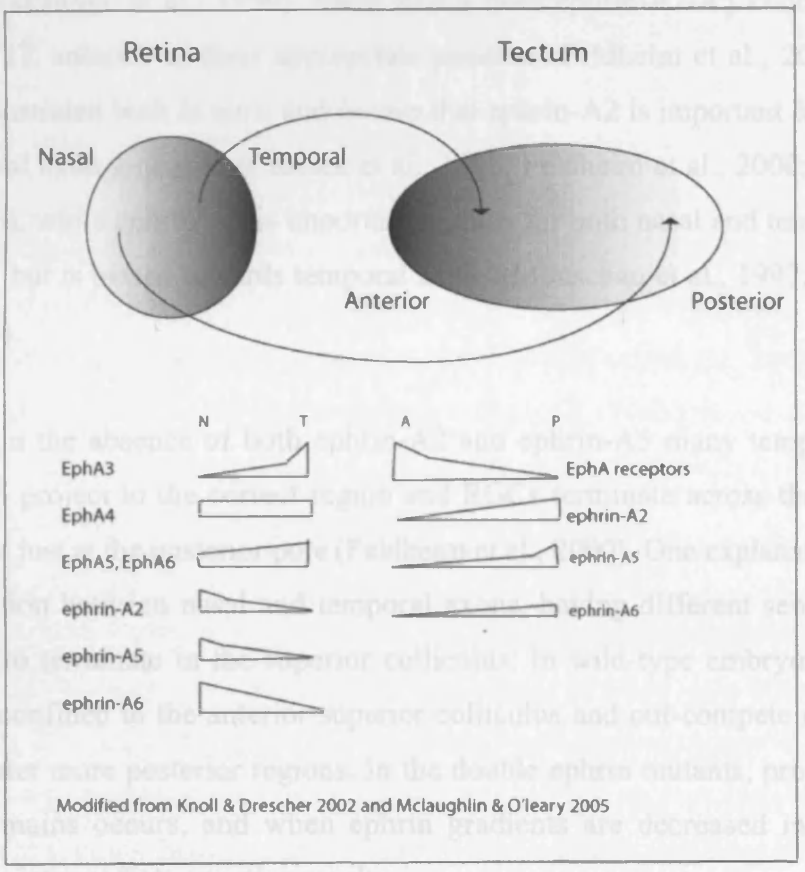


Figure legend 1.7 - Axons from the nasal and temporal retina terminate in the posterior and anterior tectum respectively. EphA receptors are expressed in a low nasal to high temporal gradient, and ephrin-A ligands in a high nasal to low temporal gradient in the retina. In the tectum, EphA receptors are expressed in a high anterior to low posterior gradient while ephrin-A ligands are expressed in a low anterior to high posterior gradient.

In support of this, when retinal axons grow up artificial gradients of ephrin-A ligands *in vitro*, they have been shown to stop at a fixed level of ephrin-A regardless of the steepness of the gradient (Rosentreter et al., 1998). In addition, homozygous null mutants of ephrin-A5 or ephrin-A2 or double heterozygotes, exhibit abnormal axon guidance. Temporal retinal ganglion cells lacking ephrin-A5 or ephrin-A2 project to TZ posterior to the appropriate position, while nasal RGCs are not affected (Feldheim et al., 2000; Frisen et al., 1998). Reduction of ephrin-A2 expression in the anterior tectum, using an avian retrovirus encoding ephrin-A2, resulted in the abnormal projection of RGC to TZ anterior to their target while nasal axons are not affected (Nakamoto et al., 1996). Nasal RGCs from ephrin-A2/A5 knock-out mice project to TZ anterior to their appropriate position (Feldheim et al., 2000). It has been demonstrated both *in vitro* and *in vivo* that ephrin-A2 is important for temporal but not nasal axon guidance (Ciossek et al., 1998; Feldheim et al., 2000; Nakamoto et al., 1996), while ephrin-A5 is important *in vitro* for both nasal and temporal axon outgrowth, but is biased towards temporal axons (Monschau et al., 1997; Nakamoto et al., 1996).

However, in the absence of both ephrin-A2 and ephrin-A5 many temporal axons continue to project to the correct region and RGCs terminate across the entire SC surface, not just at the posterior pole (Feldheim et al., 2000). One explanation for this is competition between nasal and temporal axons, having different sensitivities to repulsion, to terminate in the superior colliculus. In wild-type embryos, temporal axons are confined to the anterior superior colliculus and out-compete nasal axons that can enter more posterior regions. In the double ephrin mutants, projection into similar domains occurs, and when ephrin gradients are decreased in the single mutants, an intermediate situation results.

In support of this, ectopic expression of EphA3 in a subset of retinal axons, leads to neurons expressing ectopic EphA3 being more sensitive to ephrin-As and therefore biased to a more anterior projection, but collectively still fill the anterior-posterior extent of the superior colliculus. Neurons that do not ectopically express EphA3, or those that express dominant negative EphA3, induce a shift to a more posterior projection (Brown et al., 2000; Feldheim et al., 2004). This can be explained by them having been outcompeted by the axons with elevated EphA3 expression. Taken together, these results show that the relative levels of EphA expression by retinal axons underlie a graded response to ephrin gradients and that sensitivity to repulsion biases success when competing for space in the target tissue, rather than regulating the absolute length of a projection.

However, the situation is more complicated than this. Both EphA and ephrin-As are expressed on retinotectal axons. In addition to EphA3, at least four other EphA receptors are expressed uniformly across the retina (Connor et al., 1998; Hornberger et al., 1999; Marcus et al., 1996), together with a high nasal to low temporal gradient of ephrin-A5 and ephrin-A6 (Marcus et al., 1996; Menzel et al., 2001). EphA4 is expressed uniformly across the NT axis, but is normally only phosphorylated in nasal retina (Connor et al., 1998; Hornberger et al., 1999). This phosphorylation gradient corresponds to a high nasal to low temporal expression of high affinity ephrin-A ligands for EphA4 in the retina (Connor et al., 1998; Marcus et al., 1996). The overlap in expression pattern causes persistent activation of EphA4 in nasal axons (Connor et al., 1998), which desensitises axons to repulsion by exogenous ephrin-A ligands (Hornberger et al., 1999). *In vitro*, EphA4 is required for the repulsion of nasal but not temporal axons by exogenous ephrin-A5 (Walkenhorst et al., 2000). The overlapping expression pattern of EphA4 and ephrin-A5 seems to be important in the graded sensitivity of nasal retinal axons.

Ephrins also have a role in regulating the branching and arborisation of the RGCs. Ephrin-As inhibit branch formation along RGC axons in an *in vitro* branching assay (Yates et al., 2001). In addition, rat RGCs grown on alternating stripes of membranes from anterior or posterior regions of the SC only branch on membranes from their correct target regions (Roskies and O'Leary, 1994). It has also been demonstrated

that an inactivation of ephrin-As *in vivo*, using CALI (chromophore assisted laser inactivation) leads to enhanced RGC axon branching in the OT (Sakurai et al., 2002).

1.5.1.2 Patterning the dorsal-ventral axis

In the avian and mammalian systems RGCs from a specific DV position in the retina enter the OT or SC over a broad LM range and extend interstitial branches towards their appropriate TZ (Hindges et al., 2002; Simon and O'Leary, 1992). In addition to the graded expression pattern of EphA receptors and ephrin-A ligands throughout the AP axis, there is a graded expression of EphB receptors and ephrin-B ligands across the DV axis. EphB2 and EphB3 are expressed by retinal ganglion cells in a low to high DV gradient, complemented by a high to low DV gradient of ephrin-Bs (McLaughlin et al., 2003). In chick OT and mouse SC, ephrin-B1 is expressed in a low to high LM gradient (Braisted et al., 1997; Hindges et al., 2002), complemented by a high-to-low LM EphB gradient (Hindges et al., 2002).

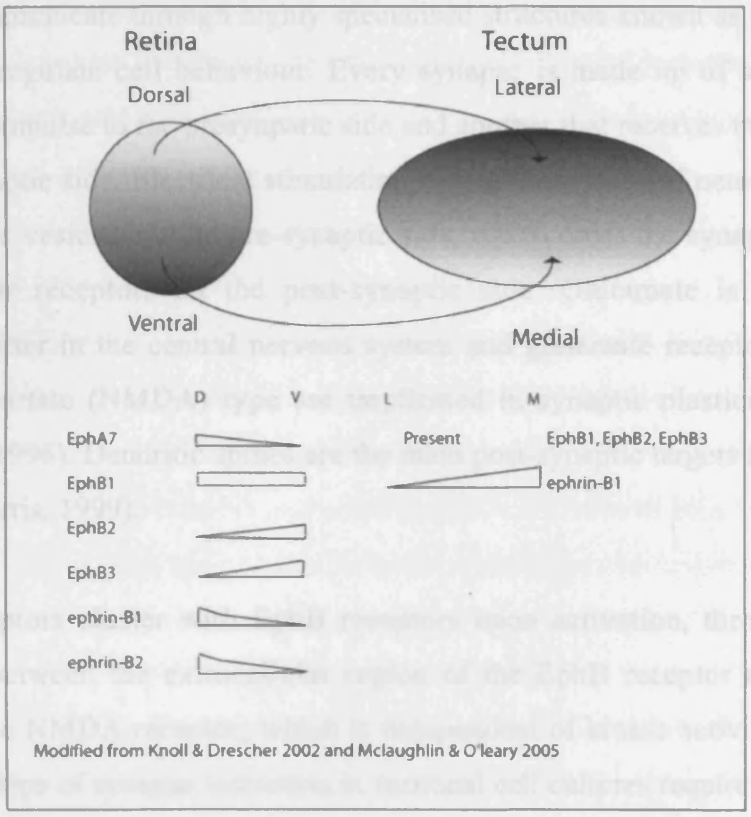
In EphB2/EphB3 null mice RGC axons orient and grow normally towards the optic disk, but as they approach the centre of the retina, a small proportion of axons defasciculate from their neighbours, bypass the optic nerve head and extend abnormally into the opposite side of the retina. A lack of both EphB2 and EphB3 simultaneously was necessary, possibly reflecting the functional redundancy of EphBs in the retina. Interestingly, although EphB2 and EphB3 are predominantly expressed in ventral retina, RGC axons from the dorsal retina are the most severely affected. In addition, rescue of the phenotype occurs in mice that express a mutant EphB2 lacking the cytoplasmic domain, suggesting that EphB2 functions in this system in a kinase-independent manner (Birgbauer et al., 2000). EphB ectodomains have been shown to cause collapse of ephrin-B expressing neurons (Birgbauer et al., 2001; Mann et al., 2003).

Ephrin-B1 and ephrin-B2 are expressed in dorsal RGCs (Braisted et al., 1997; Marcus et al., 1996), which appear to be attracted to EphB1 expressed in the lateral tectum. In *Xenopus,* ephrin-B reverse signalling has been implicated in retinotopic mapping (Mann et al., 2002). Increasing ephrin-B2 expression in ventral RGCs

results in a lateral shift of axonal projections to tectal positions, where higher levels of EphB1 are expressed, consistent with an attractive response for retinally expressed ephrin-Bs being activated on RGC axons by EphBs expressed in the OT (Mann et al., 2002). Expression of a truncated ephrin-B2 in dorsal retina led to a medial shift also consistent with an attractive effect of ephrin-Bs acting via reverse signalling (Mann et al., 2002).

In addition to their axonal projections, RGCs that are medial to their TZ preferentially extend interstitial branches laterally, down the ephrin-B1 gradient, whereas those that are lateral to their TZ extend branches medially, up the ephrin-B1 gradient. Analyses of EphB2 and EphB3 mutant mice, with and without reverse signalling intact, show abberant LM mapping due to defects in the guidance of interstitial branches. In EphB2/B3 knock-out mice branches always grow laterally down the ephrin-B1 gradient, regardless of whether the axon is lateral or medial to its TZ. In addition dominant negative EphA2 expression results in similar defects (Hindges et al., 2002). These data suggest that axons respond to an ephrin-B1 mediated attractive signal via EphB2/B3 forward signalling. In addition, the fact that interstitial branches do not go randomly in the EphB2/B3 knockouts, but all grow laterally, indicates that bi-directional branch extension requires a branch repellent in a distribution paralleling ephrin-B1 (Hindges et al., 2002; McLaughlin et al., 2003). This model is supported by the demonstration that high levels of ephrin-B1 repel interstitial branches in a selective manner (Hindges et al., 2002). These studies show that in mice and chicks ephrin-B1 acts through EphB forward signalling as both an attractant and a repellant: A branch located lateral to its nascent TZ is attracted up the gradient of ephrin-B1 towards it future TZ, whereas a branch located medial to its nascent TZ is repelled down the ephrin-B1 gradient towards its future TZ (Hindges et al., 2002; McLaughlin et al., 2003).

Figure 1.8 Gradients of Eph receptor and ephrin expression along the DV axis in the chick



Axons from the dorsal retina terminate in the lateral tectum and axons from the ventral rtetina terminate in the medial tectum. Eph receptors and ephrins are expressed in gradients thoughout the retina and tectum. EphA7 receptors is expressed in a low ventral to high dorsal gradient in the retina and EphB receptors are expressed in a low dorsal to high ventral gradient. Ephrin-B ligands are expressed in a high dorsal to low ventral gradient. In the tectum, EphB receptors are present and while ephrin-B1 is expressed in a low lateral to high medial gradient.

1.6 Eph receptors and ephrins: Roles in the adult

Eph/ephrin signalling has been shown to function widely throughout development as has already been discussed. However, signals from both Eph receptors and ephrins have also been implicated in the adult in partitioning of gut epithelia (Batlle et al., 2002), synaptic plasticity and cancer.

1.6.1 Synaptic plasticity

Neurons communicate through highly specialised structures known as synapses and in this way regulate cell behaviour. Every synapse is made up of a neuron that conducts an impulse to the presynpatic side and another that receives the stimulus at the post-synaptic side. Electrical stimulation causes the release of neurotransmitters from synaptic vesicles on the pre-synaptic side, which cross the synaptic cleft and activate their receptors on the post-synaptic side. Glutamate is the primary neurotransmitter in the central nervous system and glutamate receptors of the *N*-methyl-$_D$-aspartate (NMDA) type are implicated in synaptic plasticity processes (Katz et al., 1996). Dendritic spines are the main post-synaptic targets for excitatory synapses (Harris, 1999).

NMDA receptors cluster with EphB receptors upon activation, through a direct interaction between the extracellular region of the EphB receptor and the NR1 subunit of the NMDA receptor, which is independent of kinase activity. However, subsequent steps of synapse formation in neuronal cell cultures require EphB kinase activity (Dalva et al., 2000). Ephrin-B2 stimulation of EphB receptors was later shown to modulate NMDA-receptor-dependent calcium influx. Interestingly, in transfected neurons, this also requires the presence of the EphB2 cytoplasmic region. EphB activation led to Src tyrosine kinases mediated phosphorylation of NMDA receptors and an increase in the ability of the NMDA receptor to flux calcium in response to glutamate (Takasu et al., 2002). In addition, EphB2 can bind to and phosphorylate syndecan-2, which can induce the formation of excitatory synapses in hippocampal neurons. This leads to syndecan-2 clustering and spine formation. The association between syndecan-2 and EphB2 is dependent on the kinase activity of EphB2 (Ethell et al., 2001). Eph receptor signalling mediated by Rho GTPases has been shown to be important for dendritic spine morphogenesis. This will be discussed later (see 1.9.1.1).

Genetic studies have shed light on the role of EphB receptors in synaptic plasticity. Mice that lack EphB2 (EphB2$^{-/-}$) are viable and have normal hippocampal synapse morphology. However, EphB2$^{-/-}$ mice do show impaired hippocampal long-term

potentiation. Furthermore, in hippocampal slices derived from EphB2$^{-/-}$ mice, two forms of synaptic depression were absent. However, EphB2 kinase signalling is not required for these EphB2-mediated functions since expression of a truncated EphB2 receptor lacking kinase activity can rescue the EphB2$^{-/-}$ mutant phenotype (Grunwald et al., 2001; Henderson et al., 2001).

1.6.2 Cancer

There is evidence for the involvement of both EphB/ephrin-B and EphA/ephrin-A signalling in tumour angiogenesis during cancer progression from a variety of different sources as well as for tumour growth, survival and metastasis. Elevated expression of both Eph receptors and ephrins has been shown to be a factor in many human cancers including, lung, breast, prostate, melanoma and leukaemia (reviewed in Surawska et al., 2004).

EphA1 was the first Eph receptor to be cloned, from an erythropoietin-producing human hepatocellular carcinoma cell line, where EphA1 was more than ten-fold overexpressed suggesting a role in tumourigenesis (Hirai et al., 1987). The most studied Eph receptor in cancer is EphA2, which is upregulated in prostate, colorectal and lung tumours as well as melanomas (Heroult et al., 2005). Ephrin-A1 is expressed in tumour and tumour associated vasculature, together with EphA2 (Brantley et al., 2002; Ogawa et al., 2000). Inhibition of EphA signalling by addition of soluble EphA receptors has been shown to inhibit tumour induced endothelial cell migration in culture (Brantley et al., 2002; Cheng et al., 2002b), and reducing EphA2 levels using RNAi inhibits cellular invasiveness and VEGF-induced endothelial cell migration (Cheng et al., 2002b). Soluble EphA receptors have been shown to inhibit angiogenesis in a number of tumour models using a tumour vascular window assay, as well as tumour growth *in vivo,* by blocking endogenous EphA receptor activation (Brantley et al., 2002; Cheng et al., 2002b). Furthermore, in an *in vitro* angiogenesis assay, a dominant negative form of EphA2 receptor inhibited capillary tube formation by HUVECs (Ogawa et al., 2000).

Overexpression of EphA2 in non-transformed mammary epithelial cells induced malignant transformation and conferred tumorgenic potential (Zelinski et al., 2001). In two very recent studies the role of EphA2 has again been highlighted. The expression of an EphA2 mutant lacking the cytoplasmic domain or carrying a point mutation that inhibits its kinase activity decreased tumour volume and increased tumour apoptosis in primary tumours. The number of lung metastases was also reduced. The defects were not due to angiogenesis as there is no significant difference in tumour vessel density. In contrast the EphA mutants are defective in RhoA GTPase activation and cell migration. Therefore, receptor phosphorylation and kinase activity of the EphA2 receptor, at least in part, contribute to tumour malignancy (Fang et al., 2005). EphA2 deficient mice have also highlighted the specific role of EphA2 expression on the endothelium for the progression of breast metastasis and tumour angiogenesis (Brantley-Sieders et al., 2005).

High levels of EphA2 expression appear to be indicative of poor clinical prognosis. In a study of oesophageal squamous cell carcinoma, patients with EphA2 positive tumours had poorer survival rates compared with patients with EphA2 negative tumours (Miyazaki et al., 2003). In addition, high levels of EphA2 expression correlated with a high degree of lymph node metastasis (Miyazaki et al., 2003). EphA2 may be an important therapeutic target in the fight against cancer.

The involvement of EphB receptors and ephrin-B ligands in tumour progression has not been as well characterised as that of the EphA/ephrin-A family. However, EphB2 has been shown to be upregulated in gastrointestinal and liver tumours and is detected in ovarian, lung and renal cancers, and EphB4 is expressed in mammary, lung colorectal and ovarian tumours and has actually been identified as a potential diagnostic marker for mammary tumours (Heroult et al., 2005).

Extensive ephrin-B2 expression has been observed within the tumour vasculature of a number of mouse tumour models (Gale et al., 2001; Shin et al., 2001a). In addition, soluble Eph receptors have been used successfully in animal models to block tumour neovascularisation via EphB receptors (Martiny-Baron et al., 2004), and ephrin-B1 promotes tumour growth by initiating tumour angiogenesis in hepatocellular carcinoma cells (HCC) (Sawai et al., 2003). It has recently been shown that EphB4

may promote the growth of tumours by stimulating angiogenesis through ephrin-B2. It is proposed that EphB4 expressing tumour cells function as support cells for ephrin-B2 positive vascular cells and promote the formation of blood vessels that drive increased tumour growth (Noren et al., 2004). Ephrin-B2 reverse signalling looks likely to be involved, since overexpression of truncated EphB4 with a signalling defective cytoplasmic mutation still leads to enhanced tumour growth (Noren et al., 2004). Consistent with this, blocking bi-directional signalling between ephrin-B2 and EphB4 using a soluble EphB4 monomer inhibited A375 melanoma growth and tumour angiogenesis (Martiny-Baron et al., 2004). The levels of EphB4 correlate with the degree of tumour malignancy in transgenic mouse models of breast cancer (Andres et al., 1994; Nikolova et al., 1998), and de-regulated expression of ephrin-B2 and EphB4 normally present throughout mammary gland morphogenesis was seen in invasive metastasising mammary tumours. Ephrin-B2 expression was lost at the earliest stages of carcinogenesis and a transition of EphB4 expression from myoepithelial to tumour cells occurs as the cancer progressed (Nikolova et al., 1998). The loss of ephrin-B2 at the onset of tumourigenesis suggests that loss of ligand results in uncontrolled growth and presence of the ligand is vital for normal mammary gland morphogenesis to occur (Nikolova et al., 1998). EphB4 is thought to be an early indicator of malignant development (Berclaz et al., 2003).

EphB4 has also been implicated as a survival factor in tumour cells. Downregulation of EphB4 in prostate tumours or mesotheliomas with oligodeoxynucleotides leads to a reduction in tumour growth and angiogenesis as well as an increase of tumour cell apoptosis (Xia et al., 2005a; Xia et al., 2005b). In contrast however, EphB2 has recently been reported to be a tumour suppressor in prostate and colorectal tumours (Batlle et al., 2005; Huusko et al., 2004).

1.6.3 Wound Healing

Expression of Eph receptors and ephrins has been found at sites of would healing in a number of different models. For example, Shin and colleagues observed strong ephrin-B2 staining in what appeared to be blood vessels in wounded tissue undergoing healing (Shin et al., 2001a). In addition, a complex expression pattern of

Eph receptors and ephrins exists in the normal human adult intestinal mucosa. EphA2, EphB2 together with ephrin-A1, ephrin-B1 and ephrin-B2 are expressed in the human intestine, with other members expressed at lower levels. Upregulation of ephrin-B2 was observed in the perilesional and lesional intestinal epithelial cells of patients with inflammatory bowel disease (IBD) and activation of ephrin-B2 signalling results in acceleration of wound healing during an *in vitro* scratch wound assay, and accompanied by increased stress fibre formation and enhanced lamellipodia directed towards to the wound (Hafner et al., 2005).

1.7 The actin cytoskeleton

Eukaryotic cells move using cycles of protrusion at the front of the cell and retraction at the rear and these movements rely on the correct regulation of the actin cytoskeleton. The mechanisms of actin filament assembly and disassembly necessary for cell motility have been widely studied (reviewed in Pollard and Borisy, 2003)). It is known that actin polymerisation at the leading edge of cells drives the formation of protrusive structures, which push the membrane forward, while actin depolymerisation occurs at the rear (Pollard and Borisy, 2003). Actin stress fibres, bundles of actin-myosin contractile filaments, attach to the matrix via focal adhesions and lead to shortening/contraction of the cell body to pull the cell along (Cramer, 1999).

1.7.1 The Rho family of GTPases

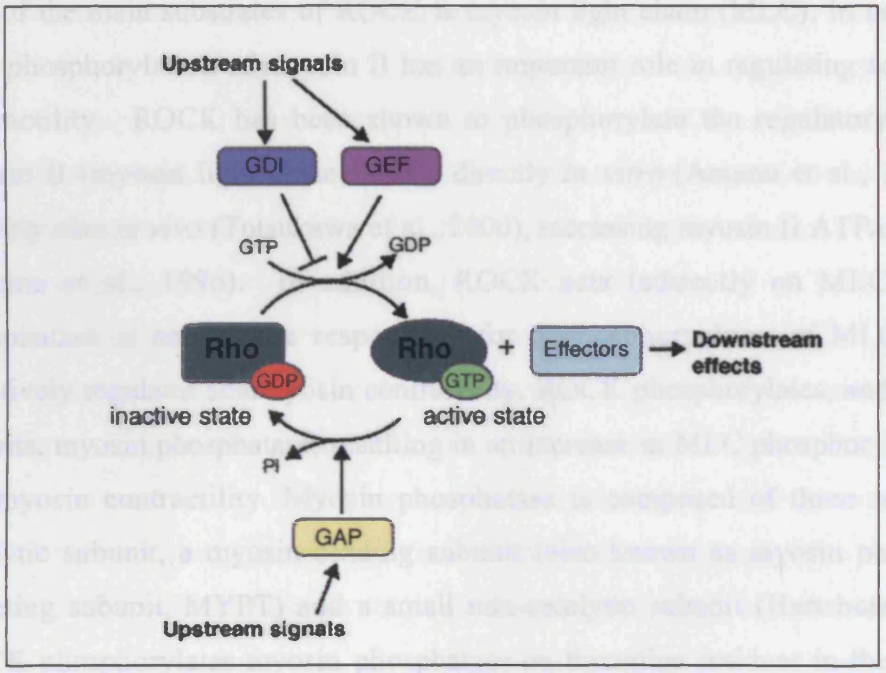
GTPase switches control a wide variety of signal transduction pathways in eukaryotic cells. Mammalian cells contain hundreds of GTPases and members of the Ras superfamily have been shown to regulate many aspects of cell behaviour. There are over 60 of these small monomeric GTPases known in mammals and they can be divided into five major groups: Ras, Rho, Rab, Arf and Ran.

The Rho family of small GTPases are important regulators of the actin cytoskeleton. 22 mammalian Rho GTPases have been described but the three best-characterised members of the family are Rho, Rac and Cdc42. Rho GTPases are small (21kDa)

proteins that cycle between an active (GTP-bound) conformation and an inactive (GDP-bound) conformation, hydrolysing GTP to switch between active and inactive states (Fig. 1.9). The active, GTP-bound, protein binds to effector molecules and transduces intracellular signals. Three classes of protein regulate the cycle. Guanine nucleotide exchange factors (GEFs) bind the inactive form of the GTPase and catalyse the nucleotide exchange of GDP for GTP, mediating activation (Nobes and Hall, 1994; Schmidt and Hall, 2002). GTPase activating proteins (GAPs) stimulate GTP hydrolysis to GDP, which leads to inactivation (Bernards, 2003). Guanine nucleotide dissociation inhibitors (GDIs) block spontaneous activation and stabilise the inactive GDP-bound GTPase (Olofsson, 1999) (Fig. 1.9). In their active GTP-bound state Rho GTPases perform their regulatory function through a conformation-specific interaction with target (effector) proteins, over 50 of which have been identified for Rho, Rac and Cdc42.

Rho GTPases regulate a wide range of signalling pathways controlling a number of cellular processes such as cell polarity and G1 cell cycle progression. However, they are best known for their role in the regulation of actin structures via a series of well-defined signal transduction pathways that lead to both the formation (actin polymerisation) and organisation (filament bundling) of actin filaments. Activation of Rho itself leads to the formation of contractile actin-myosin filaments known as stress fibres (Ridley and Hall, 1992). Activation of Rac and Cdc42 leads to the formation of protrusive actin-rich lamellipodia (Ridley et al., 1992), and finger-like filopodia (Nobes et al., 1995), respectively (reviewed in Etienne-Manneville and Hall, 2002), requiring actin polymerisation together with stabilisation of existing filaments. This occurs by antagonising the actomyosin contraction that underlies Rho-mediated changes in the actin cytoskeleton.

Figure 1.9 Rho GTPase cycle



Rho GTPases cycle between an active GTP-bound state, and an inactive GDP-bound state, catalysed by the hydrolysis of GTP. This cycle is regulated by three classes of protein: GEFs (guanine nucleotide exchange factors) which trigger the release of GDP allowing GTP binding; GAPs (GTPase activating proteins) which catalyse GTP hydrolysis; GDIs (guanine nucleotide dissociation inhibitors) which block spontaneous activation.

The Rho induced formation of contractile actin and myosin stress fibres occurs through the Rho effector, Rho kinase (ROCK). ROCK is a serine threonine kinase and consists of an amino-terminal kinase domain, followed by a potential coiled-coil forming region. At the carboxyl-terminus there is a Rho-binding domain and a pleckstrin homology domain, which has an internal cysteine-rich region. There are two isoforms of ROCK, which have 65% overall sequence similarity, with the highest similarity (92%) found in the kinase domain (Nakagawa et al., 1996). Rho binding moderately enhances the kinase activity of ROCKs (Ishizaki et al., 1996; Matsui et al., 1996). In addition, ROCK inhibitors and dominant negative ROCK constructs inhibit Rho-induced stress fibre formation, although a complex between endogenous ROCK isoforms and Rho has not been shown (Amano et al., 1997; Uehata et al., 1997).

One of the main substrates of ROCK is myosin light chain (MLC). In non-muscle cells phosphorylation of myosin II has an important role in regulating actomyosin contractility. ROCK has been shown to phosphorylate the regulatory chain of myosin II (myosin light chain; MLC) directly *in vitro* (Amano et al., 1996) and possibly also *in vivo* (Totsukawa et al., 2000), increasing myosin II ATPase activity (Amano et al., 1996). In addition, ROCK acts indirectly on MLC. Myosin phosphatase is an enzyme responsible for dephosphoryaltion of MLC, and so negatively regulates actomyosin contractility. ROCK phosphorylates, and therefore inhibits, myosin phosphatase, resulting in an increase in MLC phosphorylation and actomyosin contractility. Myosin phosphatase is composed of three subunits: a catalytic subunit, a myosin binding subunit (also known as myosin phosphatase targeting subunit, MYPT) and a small non-catalytic subunit (Hartshorne, 1998). ROCK phosphorylates myosin phosphatase on threonine residues in the carboxy-terminal half of MYPT (Feng et al., 1999a; Kimura et al., 1996; Velasco et al., 2002). Inactivation of myosin phosphatase therefore leads to increased phosphorylation of MLC (Kimura et al., 1996), which promotes actin filament cross-linking by myosin II, leading to the assembly of contractile stress fibres and increased actomyosin contractility (Kimura et al., 1996). Therefore, Rho activation leads to an increase in cell contractility via ROCK, which is important for numerous processes such as smooth muscle cell contraction, cell adhesion and migration (reviewed in Riento and Ridley, 2003).

1.7.2 Actin polymerisation

Actin polymerisation occurs via the coordinated activities of filament severing and capping proteins, and the Arp2/3 complex and the formins are the two major actin polymerisation factors. Rac and Cdc42, together with their role in the formation of lamellipodia and filopodia respectively, initiate peripheral actin polymerisation through the Arp2/3 complex, activation of which occurs through members of the Wiskott-Aldrich syndrome protein (WASP) family. Actin related proteins, Arp2 and Arp3, form the Arp2/3 complex, an actin-nucleation complex that associates with existing actin filaments to initiate new, branched filaments (Millard et al., 2004).

Actin nucleation, site-directed branching, and elongation of actin filaments are essential for the extension of membrane protrusions such as filopodia and lamellipodia, and cell motility.

Activation of Arp2/3 by Cdc42 occurs via N-WASP. *In vitro*, GTP-bound Cdc42 binds directly to N-WASP or WASP, relieving the auto-inhibition of N-WASP and exposing a binding/activation site for the Arp2/3 complex at its C-terminus. However, N-WASP/WASP is mainly found bound to WIP (WASP interacting protein) family members such as the brain specific CR16 and this serves to stabilise its inactive conformation and suppress activation by Cdc42 *in vitro* (Ho et al., 2001). Recently however, Toca-1 (transducer of Cdc42-dependent actin assembly), a new Cdc42 target has been identified (Ho et al., 2004), which is required for activation of the N-WASP/WIP complex (Ho et al., 2004), and this then leads to activation of Arp2/3.

The WAVE family of proteins mediates activation of the Arp2/3 complex by Rac. There are thought to be two mechanisms of Arp2/3 activation by WAVEs. WAVE1 is found in a complex with HSPC300, Nap125 and PIR121 (Eden et al., 2002). Nap125 and PIR121 are direct Rac targets and it has been proposed that Rac promotes the disassembly of this inactive complex, allowing WAVE to interact with Arp2/3. A similar complex has been isolated containing WAVE2, but with Abi1 as an extra component. Unlike the WAVE1 complex, this one is active in actin polymerisation assays (Innocenti et al., 2004). It has been proposed that the role of Rac in actin polymerisation, is to localise this complex to the cell periphery and promote actin nucleation. Further studies are needed to clarify the situation.

1.8 Ephrin signalling

1.8.1 Ephrin-B signalling

Although there is evidence that both ephrin-As (Davy et al., 1999; Davy and Robbins, 2000; Huai and Drescher, 2001) and ephrin-Bs (Bruckner et al., 1997; Cowan and Henkemeyer, 2001; Lu et al., 2001) can signal into their respective cells, much more is known about the mechanisms involved in the activation and signalling of B class ligands. There are a growing number of effector molecules that have been shown to interact with ephrin-Bs and the consequent signalling pathways can be divided into two main types, phosphorylation-dependent and phosphorylation-independent.

1.8.1.1 Phosphorylation-dependent signalling

Phosphorylation of ephrin-Bs is known to occur at specific tyrosine residues situated within the C-terminal 33 residues of the cytoplasmic tail, both *in vivo* and *in vitro* (Bruckner et al., 1997; Holland et al., 1996; Kalo et al., 2001). It has been shown that this phosphorylation can occur both as a result of Eph receptor binding (Bruckner et al., 1997; Cowan and Henkemeyer, 2001) and through interactions with other receptor tyrosine kinases (Bruckner et al., 1997).

Stimulation of ephrin-B phosphorylation by EphB receptors is thought to be mediated by Src family kinases. Ephrin-B1 has been shown to be phosphorylated by Src *in vitro* (Bruckner et al., 1997; Palmer et al., 2002), and Src family kinases and ephrin-B ligands co-localise in discrete signalling centres within lipid rafts after Eph receptor induced clustering (Palmer et al., 2002).

The cytoplasmic domain of ephrin-B1 can also be phosphorylated, on tyrosine residues, by platelet derived growth factor (PDGF) receptors in the absence of Eph receptor binding, suggesting crosstalk between ephrin signalling and signalling cascades activated by other tyrosine kinases (Bruckner et al., 1997). In addition Tie-2 has been shown to directly phosphorylate the cytoplasmic domain of ephrin-B2 and

may play a role in the Eph/ephrin interactions that occur during endothelial cell communication throughout angiogenesis (Adams et al., 1999).

The FGF receptor has been shown to directly bind to and phosphorylate transmembrane ephrin-Bs *in vivo* and *in vitro*, and modulate their effect on cell adhesion. In *Xenopus* embryos, FGF receptor activation can inhibit the cell dissociative effects of x-ephrin-B1 (Chong et al., 2000; Jones et al., 1998). Also, during retinal development in *Xenopus*, FGF modulation of ephrin-B reverse signalling is required to regulate the positioning of retinal progenitors within the eye field (Moore et al., 2004).

In order to transduce reverse signals ephrin-Bs associate physically with other cytoplasmic proteins, and at least one protein containing a Src homology 2 (SH2) domain has been shown to be involved. The SH2/SH3 adaptor protein Grb4 is known to bind to the cytoplasmic domain of ephrin-B1 in a phosphotyrosine dependent manner (Cowan and Henkemeyer, 2001). In Baby Hamster Kidney (BHK) cells activation of ephrin-B1 regulates loss of actin stress fibres and after long periods of exposure the cells round up, indicating adhesive contacts are lost. There is also a redistribution of paxillin, which is lost from focal contacts, and an increase in FAK catalytic activity. These effects can be blocked by a dominant negative Grb4 construct, indicating involvement of this adaptor protein in the propagation of the reverse signal. Grb4 has been shown to bind to a number of proteins involved in cytoskeletal regulation via its SH3 domains (Cowan and Henkemeyer, 2001), such as cyclase associated protein (CAP), which has been proposed to regulate actin stress fibres and focal adhesions (Ribon et al., 1998). In mammalian cells the tyrosine residue important for Grb4 binding is Tyr304 (Su et al., 2004), and in *Xenopus* the equivalent residue is Tyr298 (Bong et al., 2004).

Recently, ephrin-B1 was found to create an *in vivo* complex with claudin1 (CLD1) and claudin4 (CLD4) via the extracellular domains of these proteins (Tanaka et al., 2005). This is the first report of an ephrin-B ligand binding a protein, other than its cognate Eph receptor, via its extracellular domain. EphB2 and claudins may competitively associate with the extracellular domain of ephrin-B1 (Tanaka et al., 2005). Claudins are the major constituents of the tight junctions of epithelial cells,

which serve as barriers to restrict the movement of ions and proteins across cell boundaries. Claudin was found to associate with ephrin-B1 *in cis* (on the same cell surface) but not *in trans* and the association of claudin with ephrin-B1 induced phosphorylation of cytoplasmic tyrosine residues, which was enhanced by cell-cell contact. The claudin-ephrin-B1 complex attenuates cell-cell adhesion through the phosphorylated cytoplasmic domain of ephrin-B1 (Tanaka et al., 2005). Phosphorylation allowed binding of the adaptor protein Grb4 and most probably occurred via Src family kinases (Tanaka et al., 2005).

1.8.1.2 Phosphorylation-independent Signalling

The PDZ binding domain at the C-terminus of the B ephrin cytoplasmic tail is important for the recruitment of numerous proteins that have been shown to be involved in the signalling cascades downstream of ephrins.

Several binding partners of ephrin-Bs have been found including FAP-1 (PTP-BL), PHIP, Syntenin (Lin et al., 1999), GRIP (Bruckner et al., 1999; Lin et al., 1999; Torres et al., 1998), GRIP-2 (Bruckner et al., 1999), and PICK1 (Torres et al., 1998). Some of these PDZ domain interactions are important for the correct localisation of transmembrane proteins, such as NMDA receptors and potassium channels at synaptic sites (Torres et al., 1998). Since clustering of ephrins and Eph receptors and localisation at sites of cell-cell contact is known to be necessary for their activation, PDZ domain associations with the carboxyl-terminus of ephrin-Bs may play a role in presenting the ligand in the correct oligomeric state, in order to elicit specific responses from the receptor (Lin et al., 1999). Co-localisation of GRIP and ephrin-B2 (and ephrin-B1, but to a lesser extent), has been shown on dorsal aorta and aortic branches and suggests a possible role for ephrin-GRIP interactions in angiogenesis (Bruckner et al., 1999).

Another PDZ domain containing protein implicated in reverse signalling is the cytoplasmic protein PDZ-RGS3, that can regulate heterotrimeric G protein signalling (Lu et al., 2001). The role of the RGS domain is to act as a GTPase-activating protein (GAP) for the α subunits of trimeric G proteins, catalysing hydrolysis from

GTP to GDP, inactivating signalling. In cerebellar granule cells, chemotaxis mediated by the G-protein coupled chemokine receptor CXCR4 is inhibited in response to EphB receptor binding to ephrin-B1, presumably though the activity of PDZ-RGS3 (Lu et al., 2001). SDF-1 (stromal-cell derived factor 1), the ligand for CXCR4, functions as a chemoattractant and is thought to promote cerebeller granule cell migration towards the fine vascular membrane covering the brain. It has been proposed that ephrin-B reverse signalling mediated by PDZ-RGS3 may act to neutralise CXCR4 attraction to SDF-1, and allow inward migration of cerebellar granule cells *in vivo* (Lu et al., 2001).

An interesting idea has been proposed concerning the PDZ containing protein PTP-BL. This protein interacts with ephrin-B1 via its fourth PDZ domain. After Src activation and ephrin phosphorylation resulting from EphB/ephrinB binding, signalling pathways are activated downstream of these molecules. Subsequently, ephrin-B clusters recruit PTP-BL through its PDZ domain. This initiates dephosphorylation of both Src and ephrinB, effectively turning off signalling. This may not completely terminate ephrin-B signalling, but rather switch it from phosphotyrosine dependent to PDZ domain dependent (Palmer et al., 2002). In this way PTP-BL is thought to function as a negative regulator of B-ephrin reverse signalling.

The MAP kinase JNK has been identified as an important mediator of ephrinB1 signalling and again seems to point to a role for reverse signalling in rearrangements of the actin cytoskeleton and cell adhesion. Overexpression of ephrin-B1 in 293T cells dramatically increases the activity of JNK. Ephrin-B1 reverse signalling resulted in a rounded cell morphology and this was found to be dependent upon JNK activity and independent of phosphorylation (Xu et al., 2003).

Recruitment of dishevelled may also be important in loss of cell adhesion/cell repulsion. *Xenopus* Dishevelled has been shown to form a complex with ephrin-B1 via binding directly to the C-terminal region of ephrin-B1, or indirectly through interaction with Grb4, upon tyrosine phosphorylation of ephrin-B1 (Tanaka et al., 2003) and interaction with Dishevelled is required for ephrin-B1-mediated cell sorting in *Xenopus* embryos, mediated by RhoA and ROCK activity. A recent study

has also reported a role for ephrin-B1 in the control of cell migration to the eye field during *Xenopus* development (Lee et al., 2006). This ephrin-B1 mediated cell movement ocured via the PCP pathway and required Daam1, PKC∂, RhoA and JNK activity downstream from the ligand (Lee et al., 2006).

1.8.1.3 Internalisation of ephrin-Bs

A recent study from our laboratory has described a novel mechanism for cell-cell-repulsion where Rac activation is triggered upon contact of an EphB4 receptor expressing cell, with an ephrin-B2 expressing cell, this causes localised membrane ruffling at contact sites followed by cell-cell retraction concomitant with endocytosis of activated Eph receptor and bound full-length ephrin-B ligand into the receptor expressing cell (Marston et al., 2003). Another group has also provided evidence for bi-directional endocytosis of both activated EphB1 and ephrin-B1, either by presentation of their respective signalling partners in soluble forms, or as a result of cell-cell contact (Zimmer et al., 2003). Interestingly, co-localisation of EphB-ephrin-B clusters with markers of the endocytic pathway such as clathrin were not found (Marston et al., 2003; Zimmer et al., 2003).

Recently, an independent study has reported reverse endocytosis of activated ephrin-B1, via a clathrin dependent pathway (Parker et al., 2004). Internalisation of both receptor and ligand into their respective cells was found upon contact of an EphB1 and an ephrin-B1 expressing cell. Reverse endocytosis of ephrin-B1 was also found upon stimulation of ephrin-B1 expressing cells with soluble EphB1-Fc. Co-expression of a dominant negative dynamin construct with ephrin-B1, or potassium depletion, resulted in reduced internalisation (Parker et al., 2004).

1.8.2 Ephrin-A signalling

Unlike the ephrin-B subclass, ephrin-A ligands are anchored to the membrane by a GPI linkage and as such do not possess an intracellular domain. However, signalling via ephrin-A ligands has been shown to occur, binding of EphA ectodomains to ephrin-A ligands can trigger a cellular response (Kullander and Klein, 2002). In

C.elegans the only known Eph receptor is VAB-1, and it is expressed together with four potential ephrin-As. Genetic studies indicated that many of the functions of VAB-1 were kinase-independent, suggesting the presence of reverse signalling by *C.elegans* ephrins (Wang et al., 1999). Activation of ephrin-A5 by EphA5 stimulation led to an increase in integrin-mediated adhesion, dependent on the Fyn tyrosine kinase (Davy et al., 1999; Huai and Drescher, 2001) and an increase in MAP kinase activation (Davy and Robbins, 2000). Furthermore activation of ephrin-A1 inhibited T-cell chemotaxis by increasing Rho activation (Sharfe et al., 2002).

The mechanism of ephrin-A signal transduction is not well understood, but it is possible that their GPI anchorage may localise them to membrane rafts rich in signalling complexes (Sargiacomo et al., 1993; Shenoy-Scaria et al., 1994) and a raft-localised potential co-receptor has been implicated (Huai and Drescher, 2001). In addition cleavage of ephrin-As by the metalloprotease ADAM10 may provide a mechanism for ephrin-A induced cell repulsive events (Hattori et al., 2000).

1.9 Eph receptor signalling

Throughout development, the biological functions of Eph receptors and ephrins involve the control of cell shape and movement, which depend on co-ordinated changes in the cell cytoskeleton and cell adhesion. *In vitro*, activation of Eph receptors has been shown to impinge on the actin cytoskeleton and cell-matrix adhesion in a variety of situations.

1.9.1 Eph receptor regulation of actin dynamics via Rho GTPases

As discussed above, the small GTPases Rac and Cdc42 are known to regulate protrusive structures known as lamellipodia and filopodia respectively, whereas Rho is important for the formation of actin stress fibres and cell contractility. Many of the responses to Eph receptor activation are repulsive in nature, therefore it may be expected that the predominant family member to feature downstream of Eph receptors is Rho. Interestingly, Rac/Cdc42 have also been implicated in Eph receptor signalling.

1.9.1.1 Rho GTPases downstream from EphB receptors

EphB receptors have been shown to interact with exchange factors for Rho family GTPases. In hippocampal neurons EphB2 signals to Rac1 and Cdc42 by binding the exchange factors Kalirin and Intersectin respectively (Irie and Yamaguchi, 2002; Penzes et al., 2003), and regulates dendritic spine morphogenesis.

Endogenous EphB2 activation in hippocampal neurons, increases Cdc42 levels as a result of the functional interaction between Intersectin and EphB2. Expression of a kinase dead EphB2, or truncated Intersectin, lacking GEF activity inhibits the formation of dendritic spines (Ethell et al., 2001; Irie and Yamaguchi, 2002). Kalirin is a member of the Trio family of GEFs, which is expressed predominantly in the nervous system, and also plays a role in dendritic spine morphogenesis (Penzes et al., 2001). Both EphB2 activation by ephrin-B1 in hippocampal neurons, and overexpression of kalirin, results in an increased number and size of dendritic spines (Penzes et al., 2003; Penzes et al., 2001), and changes in spine morphology were found to be both Eph receptor and Rac-dependent (Penzes et al., 2003). Ephrin-B1 stimulation of hippocampal neurons results in Kalirin phosphorylation on tyrosine and upregulates activation of the serine/threonine kinase PAK, a downstream effector of Rac1, specifically in dendritic spines (Penzes et al., 2003). It was actually determined that EphB2 activation triggers the redistribution of Kalirin to EphB2 membrane clusters in the post-synaptic site, and in this way promotes dendritic spine morphogenesis, rather than changing Kalirins GEF activity towards Rac (Penzes et al., 2003).

Another GEF for Rac1 and Cdc42 is αPix (Pak interacting exchange factor), which associates with activated EphB2, probably mediated by the adaptor Nck, which binds both to Pak and EphB receptors (Becker et al., 2000; Holland et al., 1997; Stein et al., 1998a). It has also been shown that Rac1, together with the adaptor protein Crk is required for cell spreading and membrane ruffling in human aortic endothelial cells treated with ephrin-B1 (Nagashima et al., 2002). Crk involvement in this Rac1-mediated process suggests the involvement of Dock180, a newly discovered Rac1 GEF that associates with Crk (Brugnera et al., 2002; Cote and Vuori, 2002).

EphB activation, as well as inducing Rac1 activation, has also been shown to decrease Rac1 activity in intestinal epithelial cells (Batlle et al., 2002). It remains to be determined whether EphB receptors signalling is cell type dependent or if Rac1 inactivation is only transient, as has been shown to be the case downstream of EphA receptors (Jurney et al., 2002).

In addition to a role for Rac1 and Cdc42 downstream from EphB receptors, there is evidence to suggest RhoA activation can occur downstream from EphB receptors. *Xenopus* Dishevelled has been shown to associate with EphB1 and EphB2, probably through an SH2 domain-containing adaptor like Nck or Grb4, although this has not been determined, and is phosphorylated on tyrosine in cells where EphB2 is activated (Tanaka et al., 2003). Ephrin stimulation of EphB2 promotes activation of RhoA and ROCK, which is inhibited by dominant negative Dishevelled and contributes to the sorting of EphB2 expressing cells from ephrin-B1 expressing cells in *Xenopus* animal cap assays. Another possible connection between EphB receptors and RhoA is through the GTPase-activating protein p120RasGAP, which binds to activated EphB receptors and also associates with p190RhoGAP (Holland et al., 1997).

1.9.1.2 Rho GTPase activation downstream from EphA receptors

In neurons, RhoA activation is known to mediate growth cone collapse and inhibit axon regeneration (Dergham et al., 2002; Fournier et al., 2003; Lehmann et al., 1999). In addition, Eph receptor activation has also been shown to regulate growth cone collapse (Cheng et al., 2003; Wahl et al., 2000). It is therefore not surprising that there is a functional link between Rho and Eph receptors. Stimulation of EphA receptors in cultured retinal ganglion cells (RGCs) with ephrin-A5 induces Rho-ROCK dependent growth cone collapse (Wahl et al., 2000). Interestingly, addition of ephrin-A5 also decreases the amount of active Rac, indicating that the activation state of both these GTPases is influenced by ephrin-A5. Decreasing Rac or Cdc42 results in retraction of lamellipodia and filopodia, which could potentiate the repulsive signal (Jurney et al., 2002; Wahl et al., 2000). However, Rac1 activity is only transiently downregulated by ephrin-A ligands in cultured RGCs (Jurney et al.,

2002). Resumption of Rac is actually necessary for reorganising actin filaments to the centre of the collapsing growth cone during ephrin-A2 induced growth cone collapse (Jurney et al., 2002), and for promoting actin polymerisation that drives membrane internalisation by endocytosis (Fournier et al., 2000).

Regulation of GTPases by Eph receptors to achieve the correct guidance response occurs via the activity GEFs and GAPs for Rho family proteins. In neurons, Ephexin, a GEF that activates RhoA and to a lesser extent Cdc42, binds constitutively to the kinase domain of EphA4 (Shamah et al., 2001). Binding to inactive EphA receptors may regulate the membrane localisation of Ephexin, and therefore RhoA and Cdc42 activity, at sites of EphA receptor expression. The catalytic activity of Ephexin is increased upon EphA receptor activation, resulting in enhanced RhoA activation in cortical neurons. Concomitantly, Rac1 and Cdc42 activity are downregulated as described earlier in response to EphA activation in retinal neurons (Jurney et al., 2002; Wahl et al., 2000). Ephexin is highly expressed in RGCs during the establishment of their topographical connections in the visual centres of the brain, suggesting a role in guiding these axons to their targets (Shamah et al., 2001).

An exchange factor closely related to Ephexin, and exclusively expressed in vascular smooth muscle cells, is Vms-RhoGEF. Like Ephexin, Vms-RhoGEF binds constitutively to EphA4 and mediates RhoA activation in rat vascular smooth muscle cells stimulated with ephrin-A1-Fc (Ogita et al., 2003), resulting in actin stress fibre assembly, probably leading to vascular contractility. EphA4 overexpression does not substantially activate RhoA in HEK 293 cells unless Vms-RhoGEF is also expressed (Ogita et al., 2003), indicating that the ability to activate RhoA depends on the presence of this exchange factor. Interestingly, presenting ephrin-A1 immobilised on a substrate rather than as a pre-clustered soluble protein, inhibits Rac1, and this inhibition is critical for the morphological effects induced by immobilised ephrin-A1 in rat vascular smooth muscle cells (Deroanne et al., 2003). It has been shown that EphA4 phosphorylates Vms-RhoGEF upon ephrin-A1 binding (Ogita et al., 2003), so regulation of these exchange factors may occur by tyrosine phosphorylation.

RhoA activity downstream from EphA receptors has also been demonstrated in non-neuronal cells. Stimulation of EphA3 expressing 293T cells with ephrin-A5 causes

activation of RhoA, leading to retraction of cellular protrusions, cell rounding and membrane blebbing. This effect appears to be mediated by the adaptor protein Crk (Lawrenson et al., 2002). In addition, stimulation of EphA expressing 293T cells with ephrin-A1 also causes activation of RhoA, while concomitantly blocking chemokine-induced activation of Cdc42, which leads to an inhibition of chemotaxis (Sharfe et al., 2003). It is not known whether exchange factors are involved in the activation of RhoA in these instances.

Another possible link between EphA receptors and RhoA is via the phosphatase Shp-2. In PC-3 prostate epithelial cells Shp-2 is recruited to activated EphA2 (Miao et al., 2000), and negatively regulates RhoA activation (Schoenwaelder et al., 2000).

1.9.2 Eph receptor regulation of cell-matrix adhesion

Integins are transmembrane heterodimers that link the extracellular matrix (ECM) to the cytoskeleton. Engagement of integrins by ECM proteins results in the recruitment of signalling molecules at sites of cell-matrix adhesion, and activation of so called "outside-in signalling". Integrin-mediated adhesion can be influenced by intracellular signals and this is known as "inside-out" signalling. Eph receptors are known to regulate cell adhesion through integrins, and this can modify cell adhesion (Huynh-Do et al., 1999; Huynh-Do et al., 2002).

EphB1 activation seems to result in an increase in integrin-mediated cell-matrix attachment. For example, EphB1 signalling enhances $\alpha 5\beta 1$ integrin-mediated cell attachment in transfected human kidney cells, and $\alpha_V\beta_3$ integrin mediated attachment in endothelial cells endogenously expressing EphB1, a process which required EphB1 kinase activity, recruitment of low molecular weight protein tyrosine phosphatase (LMW-PTP) and the SH2-SH3 adaptor protein Nck (Huynh-Do et al., 1999). In addition, downstream activation of the Nck-interacting kinase (NIK) couples EphB1 to activation of integrins and increased cell attachment (Becker et al., 2000).

Integrin-mediated increases in cell attachment have also been reported downstream from other Eph receptors. The EphA8 receptor, independently of tyrosine kinase activity, can localise the p110γ regulatory subunit of phosphatidylinositol 3-kinase (PI3Kγ) to the plasma membrane, which has been shown to be required for integrin-mediated cell adhesion (Gu and Park, 2001). In addition, ephrin-A3 stimulation of EphA2 dendritic cells has recently been shown to result in an increase in beta1 integrin-mediated attachment to fibronectin (de Saint-Vis et al., 2003), and furthermore, overexpression of ephrin-B2 in B16 mouse melanoma cells results in constitutive activation of FAK, and a significant increase in beta1-integrin-mediated attachment to the matrix (Meyer et al., 2005).

EphB1 activation induces the formation of a protein complex composed of RasGAP and phosphorylated p62DOK, which can bind to and inactivate RasGAP (Becker et al., 2000; Kashige et al., 2000). Since RasGAP can downregulate R-Ras (Rey et al., 1994) and R-Ras activity can be correlated with an increase in cell-matrix adhesion (Kinashi et al., 2000; Kwong et al., 2003), it is possible that EphB1-mediated phosphorylation of p62DOK inhibits RasGAP, and the resulting increase in R-Ras activity leads to an increase in cell-matrix adhesion.

In contrast, decreased cell adhesion has been reported downstream from EphB2. Activation of EphB2 by multimerised ephrin-B1, or by receptor overexpression, inhibits cell adhesion through phosphorylation of R-Ras, which suppresses the ability of R-Ras to support integrin activity (Zou et al., 1999), and binding of SHEP1 (SH2 domain-containing Eph receptor-binding protein 1) provides a physical link between R-Ras and EphB2 (Dodelet et al., 1999). In addition, overexpression of EphB2 in glioma cells was recently found to result in reduced cell adhesion, mediated by R-Ras activation (Nakada et al., 2005). The involvement of the small GTPase R-Ras is not surprising since increased cell-matrix adhesion is known to occur upon activation of the small GTPase R-Ras and inhibition of R-Ras prevents cells from maintaining integrin-mediated attachment (Kinashi et al., 2000; Kwong et al., 2003).

Once again, decreased cell attachment as a consequence of Eph receptor activation is not restricted to one receptor. Activation of endogenous EphA2 in prostate carcinoma cells suppresses integrin-mediated spreading, migration and adhesion, by

inducing dephosphorylation of focal adhesion kinase (FAK) and paxillin, which leads to reduced adhesion (Miao et al., 2000). FAK dephosphorylation is likely to be mediated by the phosphatase SHP2, which is recruited to EphA2 following ephrin-A1 stimulation (Miao et al., 2000). In addition, ephrin-B1 stimulation of LS174T colorectal epithelial cells endogenously expressing EphB receptors inhibits integrin-mediated adhesion (Miao et al., 2005) Using 293 cells stably transfected with wild-type or kinase deficient EphB3 inhibition of integrin-mediated attachment was found to be kinase-dependent (Miao et al., 2005).

The decision as to whether signals downstream from Eph receptor/ephrin signalling result in an increase or decrease in cell-matrix attachment, seems to be mediated by the small GTPase R-Ras. Eph receptor activation of R-Ras, via p62DOK-mediated inhibition of RasGAP, increases integrin-mediated attachment via FAK and possibly PI3-kinase. In contrast, direct phosphorylation of R-Ras by Eph receptors inhibits FAK phosphorylation, and recruits SHP to dephosphorylate FAK, reducing integrin-mediated adhesion.

1.9.3 Other Eph receptor binding partners and influences

As detailed previously (1.3), Eph receptor-ligand binding results in receptor dimerisation and subsequent transphosphorylation by the kinase domains of the two receptors. Phosphorylation is known to occur on multiple tyrosine residues within the kinase domains of the receptor and these phosphorylated tyrosine residues serve as docking sites for the SH2 domain of many signalling proteins. Molecules known to bind to Eph receptors include the adaptors Grb7 (Han et al., 2002), Grb10 (Stein et al., 1996), Nck and Crk (Hock et al., 1998a; Stein et al., 1998a), RasGAP, (Hock et al., 1998a; Holland et al., 1997), the Src family of non-receptor tyrosine kinases (Ellis et al., 1996; Zisch et al., 1998) and Abl (Yu and Bargmann, 2001). Work from our lab has recently implicated Abl family kinases in EphA receptor-mediated growth cone collapse (Harbott and Nobes, 2005). Abl is known to interact with other proteins implicated in Eph receptor signalling such as Src (Plattner et al., 1999), p62DOK and RasGAP (Yamanashi and Baltimore, 1997) and Crk (Feller et al., 1994). In addition, many interactions with PDZ domain-containing proteins have

been reported such as with AF6, TRK interacting protein, Pick I, Syntenin (Hock et al., 1998b). However, the physiological relevance of many of these interactions is unknown.

The role of Eph receptors and ephrins in blood vessel development has been discussed previously (see 1.4). Studies in endothelial cells have shed light on the downstream signalling pathways involved in the migration of endothelial cells. It is known that primary human microvascular endothelial cells, endogenously expressing EphB4, show a positive chemotactic response to ephrin-B2, and this response is blocked by inhibiting Src kinase activity (Steinle et al., 2002). Similarly, ephrin-B2 stimulation of a cell line that endogenously expresses EphB1 leads to a Src-dependent chemotactic response (Vindis et al., 2003). In both cell types Src becomes phosphorylated on tyrosine 416, which is indicative of Src tyrosine kinase activity (Kmiecik et al., 1988; Steinle et al., 2002; Vindis et al., 2003). EphB1 and EphB2 have been shown to interact with Src, and EphA4, EphA8 and EphB3 can all associate with the closely related tyrosine kinase Fyn (Choi and Park, 1999; Ellis et al., 1996; Hock et al., 1998a; Prevost et al., 2002). Cell migration mediated by EphB1 and EphB2 in these systems was also shown to be dependent on the activities of MEK and PI-3 kinase respectively, and Src is required for activation of both of these pathways downstream of Eph receptor activation (Steinle et al., 2002; Vindis et al., 2003).

In addition to the regulation of Rho GTPases, Eph receptors and ephrins also regulate Ras family proteins (reviewed in Noren and Pasquale, 2004)). Both EphA and B receptors are known to negatively regulate H-Ras and its downstream MAP kinase pathway in a variety of cell types (Elowe et al., 2001; Miao et al., 2001; Nagashima et al., 2002). In addition, activation of MAP kinases occurs downstream from Eph receptors (Pratt and Kinch, 2003; Zisch et al., 2000). Activation of R-Ras and Rap1 has also been shown as a result of Eph receptor activation (Nagashima et al., 2002; Zou et al., 1999).

1.10 Regulation of repulsion versus adhesion

Evidence shows that Eph receptors can mediate both repulsion and adhesion, but the mechanisms by which this is possible are not well clarified. Eph/ephrin signalling can trigger both repulsive and adhesive responses in the same cells. Secondly, even though both Eph receptors and ephrins are membrane bound, and therefore require direct cell-cell contact for signalling to occur, they are able to mediate cell repulsion.

1.10.1 Endocytosis of EphB receptors and ephrin-Bs

EphB/ephrin-B signaling has been shown to result in cell repulsive events. An important question therefore was how two membrane-tethered proteins could result in cell-cell separation events, following high affinity binding of Eph receptor and ligand. Recent work from our lab has shown that Rac dependent trans-endocytosis of EphB-ephrin-B complexes is necessary for separation of cells following receptor-ligand interaction at sites of cell-cell contact (Marston et al., 2003). A recent study from Klein and colleagues has also proposed that bi-directional endocytosis of full-length Eph receptors and ephrins may be a mechanism allowing EphB/ephrinB dependent contact repulsion. They demonstrate that full-length receptors or ligands are transferred into the opposing cell and this removes the Eph/ephrin complex from the sites of cell-cell contact (Zimmer et al., 2003). It is possible that regulation of EphB receptor/ephrinB endocytosis will be an important modulator of Eph/ephrin attraction or repulsion.

1.10.2 Different splice forms of Eph receptors

One suggested explanation for the multiple signalling outcomes is that different splice forms of the receptor result in different effects. Different splice forms of EphA7 have been shown to ellicit different effects upon ephrin-A5 binding (Holmberg et al., 2000). In neuronal projections, ephrin-A5 null mice have revealed the importance of ephrin-A5 mediated repulsion for establishing the correct topography during neural development (Feldheim et al., 1998; Feng et al., 2000; Frisen et al., 1998; Vanderhaeghen et al., 2000). In addition, cells expressing ephrin-

A5 induce a repulsive response to EphA7 (Holmberg et al., 2000). However, defects in cell adhesion were observed upon expression of an EphA7 splice variant lacking the tyrosine kinase domain, converting the response from a repulsive one into an adhesive one (Holmberg et al., 2000). Different splice forms of an Eph receptor can determine whether a cell responds to its ligand with repulsion or adhesion.

1.10.3 Cleavage of ephrin-As

A mechanism has recently been put forward to explain how membrane-tethered proteins can regulate cell-repulsion and axon withdrawal. It was found that EphA3 stimulation of ephrin-A2 expressing neuroblastoma cells resulted in the shedding of the extracellular domain of ephrin-A2 into the medium (Hattori et al., 2000), and this cleavage was essential for cell-cell separation, and was mediated by the metalloprotease ADAM10/kuzbanian (Hattori et al., 2000).

This finding, together with the discovery of a truncated isoform of ephrin-A1 that inhibits cleavage of the full-length ephrin (Finne et al., 2004) suggests that regulation of ephrin-A cleavage may be important for switching between a repulsive and adhesive response. The constitutive association of ADAM10 with ephrins raises the possibility that cleavage-independent receptor binding is also possible.

1.11 Aims

As described previously both Eph receptors and their ephrin ligands are membrane-bound. Therefore in order for signalling to occur direct cell-cell contact is required. It is well known that upon receptor-ligand binding a signal is generated through the receptor-bearing cell. However, more recently it has been shown that a signal can also be generated via the ligand-bearing cell, known as reverse signalling. Many downstream effects of both Eph and ephrin signalling have been described. However, much more is known about the signalling pathways downstream from the Eph receptors than their ligands the ephrins.

Reverse signalling through ephrin-Bs has been described to play a role in many processes throughout development such as development of the blood (Heroult et al., 2005) and lymphatic systems (Makinen et al., 2005), boundary formation (Mellitzer et al., 1999; Xu et al., 1999), and axon guidance in the visual system (Birgbauer et al., 2000; Birgbauer et al., 2001) and central nervous system (Henkemeyer et al., 1996). The aim of this work was to investigate *in vitro,* using two model systems, (Swiss 3T3 fibroblasts exogenously expressing ephrin-B2, and HUAECs (Human Umbilical Arterial Cells) endogenously expressing ephrin-Bs), the morphological changes triggered as a result of ephrin-B activation by treatment with soluble pre-clustered EphB receptors, and to dissect the signalling pathways involved downstream from the ephrin-B ligands. Since many of the effects observed as a result of EphB forward signalling are known to impinge directly on the actin cytoskeleton the role of ephrin-B signalling in actin cytoskeletal regulation and the potential involvement of the Rho family of GTPases, downstream from ephrin-B activation was investigated.

Chapter 2

Materials and Methods

2.1 Cell culture

2.1.1 Preparing glass coverslips

To ensure consistent results, glass coverslips were cleaned, to remove any residual detergent, and baked before use. 13mm diameter glass coverslips no. 1.5 (BDH) were washed in concentrated Nitric acid (BDH) in a 1L conical flask for 10min, rinsed under running tap water for 10min, then twice in distilled water and once in sterile water (Baxter). After a final wash in methanol (BDH), that prevents the coverslips sticking together during baking, coverslips were transferred to a glass petri dish and baked at 120°C for 4h to sterilise. For microinjection, coverslips were marked with a cross at their centre using a diamond pen prior to acid washing and baking. This facilitates the localisation of injected cells after staining. Swiss 3T3 cells were plated directly onto glass coverslips. Unless otherwise indicated, HUAECs were seeded onto gelatin-coated coverslips (see 2.1.3).

2.1.2 Swiss 3T3 fibroblasts

2.1.2.1 Thawing cells

Swiss 3T3 fibroblasts were thawed from P7 stocks stored under liquid nitrogen. 15ml warmed Dulbecco's modified Eagle's medium (DMEM; Gibco), supplemented with 10% Foetal Calf Serum (FCS; PAA Laboratories) plus penicillin (100U/ml) and streptomycin (100µg/ml) (Penicillin-Streptomycin liquid; Gibco) was added drop-wise to the defrosted cell suspension in an 80cm^2 tissue culture flask (Nunc). Cells were incubated at 37°C, 10% CO_2 for a minimum of 2h, medium changed to fresh DMEM/10% FCS to remove DMSO, and incubated as above until 80-90% confluent. If necessary cells were medium changed after 3-4 days.

2.1.2.2 Culture and passaging

Swiss 3T3 fibroblasts were grown in DMEM/10% FCS and passaged once the cells reached 80-90% confluency (3-4 days). Medium was aspirated and cells were

washed twice with 3ml trypsin-EDTA (Gibco) then incubated with 0.5ml trypsin-EDTA for 1-2min at room temperature. Following trypsinisation, 9.5ml warmed DMEM/10% FCS was added to inhibit trypsin and cells were dislodged by gentle tapping. Cells were re-plated at a 1:5 dilution onto 80cm^2 flasks in DMEM/10% FCS and incubated at 37°C, 10% CO_2. Cells from passages 8-13 were used for experiments. Swiss 3T3 fibroblasts cultured beyond passage 13 increasingly showed signs of transformed colonies and were therefore not used for experiments.

2.1.2.3 Preparation of confluent, quiescent Swiss 3T3 fibroblasts

Cell number was estimated using a haemocytometer and cells were seeded on 13mm glass coverslips at high density, (5x10^4 cells per well), in 4-well multiwell dishes (Nunc) with 1ml DMEM/4% FCS. Cells were incubated at 37°C, 10% CO_2 for 7-10 days without medium change, during which time they became confluent and quiescent. Quiescent cells characteristically form a confluent monolayer with adherens junctions, evenly spaced nuclei and no evidence of cell division. These cells retain some residual actin stress fibres (see Fig. 3.1).

For some experiments quiescent Swiss 3T3 cells were serum-starved overnight (16h). 50µl of conditioned medium was removed from each well, centrifuged to remove dead cells and kept to one side. The remaining medium was replaced with 950µl serum free medium (SFM; see 2.12) plus 50µl conditioned medium. Cells were incubated at 37°C, 10% CO_2. Serum-starved Swiss 3T3 fibroblasts display few, if any, actin stress fibres and a thin band of cortical actin around their periphery (see Fig. 3.9a).

2.1.3 Human Umbilical Arterial Endothelial Cells (HUAECs)

2.1.3.1 Thawing cells

HUAECs (TCS Cellworks) were supplied as frozen P1 stocks, and stored under liquid nitrogen. One 80cm^2 flask was coated with 5ml 1% gelatin (2% gelatin diluted 50:50 with sterile H_2O; Sigma) or 5ml attachment factor (gelatin based; TCS

Cellworks) at room temperature for 2h. 30min prior to thawing cells the solution of gelatin/attachment factor was aspirated, and replaced with 19ml full medium made up of human endothelial serum free medium (Gibco), supplemented with 2% FCS, EGF (as manufacturers instructions), FGF (3μg/ml) and an antibiotic supplement comprising Gentamicin (25μg/ml) and Amphotericin (50μg/ml) (large vessel endothelial cell growth supplement; TCS Cellworks). The medium was then equilibrated at 37°C, 5% CO_2. HUAECs were thawed in a 37°C water bath, added drop-wise to the equilibrated medium and incubated overnight. The following day, cells were medium changed to fresh full medium and left for 5 days, with a medium change to fresh full medium every 48h. P1 cells were grown up, passaged to expand and re-frozen as P3 stocks (as described in 2.1.3.3).

2.1.3.2 Culture and passaging

HUAECs were cultured in full medium and passaged when cells reached 80-90% confluency (3-4 days). Medium was aspirated and cells rinsed once in 5ml buffered saline rinsing solution (TCS Cellworks), incubated with 3ml trypsin-EDTA (TCS Cellworks) for 1-2min at room temperature to allow cells to detach and resuspended in 5ml trypsin blocking solution (TCS Cellworks). The cell suspension was then spun in a swinging rotor centrifuge at 1500rpm for 5min at 4°C. Supernatant was removed and cells resuspended in 10ml full medium. Cells were plated at a 1:4 dilution onto gelatin/attachment factor coated 80cm^2 flasks containing equilibrated medium, and medium changed every 48h. Alternatively cells were resuspended in 10ml full medium as described for Swiss 3T3 fibroblasts (see 2.1.2.2) and plated as described above. HUAECs from passages 3-6 were used for experiments. After P6 cells showed increasing signs of becoming senescent, indicated by increased size, flat morphology and multiple nuclei per cell (see Fig. 4.2).

For biochemistry 1.5-2.5ml HUAECs cell suspension, (approx 4.5-7.5 x10^5 cells), was plated onto 90mm dishes (Nunc) that had been coated with 4ml attachment factor for 2h at room temperature. Cells were incubated for 2-5 days at 37°C, 5% CO_2 until the required cell density was reached. Cells were medium changed with full medium every 48h.

For cell biology, 60mm dishes (Nunc) containing 8 glass coverslips were coated with 2.5ml 1% gelatin or attachment factor for 2h at room temperature. Cells were seeded at low density to obtain sub-confluent cells (1.25 x 10^5 cells per dish for use in 24h; 0.625 x 10^5 cells for use in 48h) and at higher density (3 x10^5 cells per dish for use in 48h) for confluent cultures. Cells were medium changed with fresh full medium every 24h.

It was necessary to serum-starve HUAECs for some experiments. HUAECs grown on coverslips and 90mm dishes were serum-starved overnight (16h). Medium was replaced with human endothelial SFM in the absence of growth factors and serum.

2.1.3.3 Freezing down cells

HUAECs were frozen down at passage 3. One 90% confluent 80cm^2 flask gave 4 x 1ml frozen stocks. Cells were trypsinised and resuspended in 4ml DMEM supplemented with 50% FCS at 4°C. Dimethyl sulfoxide (DMSO; Sigma) was added drop-wise to 10% on ice with gentle mixing and 1ml suspension aliquoted per cryotube (Nunc). Cryotubes were frozen upright at -20°C overnight and transferred to -80°C for up to one week, with long-term storage in liquid nitrogen.

2.2 Cell microinjection

Typically expression plasmids (see Table 2.1) were microinjected at a concentration of 200μg/ml. DNA (prepared using Qiagen maxi-prep protocol) was diluted in sterile filtered PBS-A and injected together with dextran as a marker, (generally biotin dextran [2mg/ml; Molecular Probes]). Solutions to be injected were spun down at 13,000rpm for 5min in order to pellet any debris.

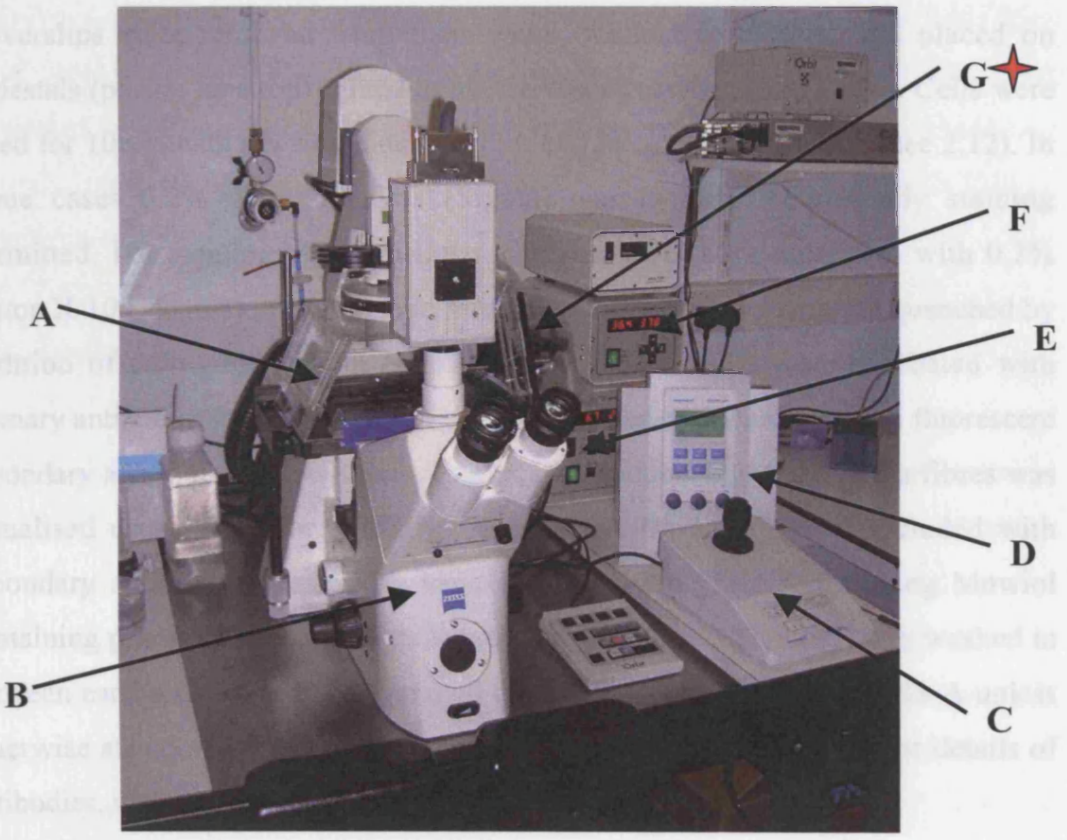
Table 2.1 Expression plasmids used for microinjection

Expression Plasmid	Source
pRK5	Nobes Lab
ephrin-B2	Ralf Adams (CRUK, London, UK)
Truncated ephrin-B2	Ralf Adams (CRUK, London, UK)
5Y-F ephrin-B2	Tony Pawson (Toronto, Canada)
ephrin-B2ΔV	Ralf Adams (CRUK, London, UK)
dn Grb4	Mark Henkemeyer (UT South Western, Texas)
C3 Transferase	Nobes Lab

For microinjection, cells grown on coverslips were placed in the centre of a 60mm dish containing 4ml equilibrated medium, placed on the heated stage of an inverted microscope (Axiovert 200M; Zeiss) and viewed with 5x, 20x and 40x phase contrast air objectives. Cells were maintained at 37°C in an enriched CO_2 environment. Boro-silicate needles (Harvard apparatus) were pulled with a horizontal needle puller (Suter P-97) and DNA was injected under constant pressure, typically 50-200hPa. Cultures were replaced in a humidified tissue culture incubator at 37°C, 5%-10% CO_2 (cell type dependent) for 2-2.5h, to allow for expression of the injected plasmid. Injections were carried out using the 40x air objective. The cross in the centre of the coverslip can be used to help re-locate the cells after staining, (see Fig. 2.1 for an overview of the microinjection apparatus).

Swiss 3T3 cells were microinjected in 4ml serum free medium at 37°C, 10% CO_2. Typically each coverslip would be injected for 15min allowing 100-150 cells to be injected. HUAECs were microinjected in 4ml full medium at 37°C, 5% CO_2, for a maximum of 10min per coverslip, after which cell morphology changes occurred. This allowed 50-70 cells per coverslip to be injected.

Figure 2.1 Microinjection Apparatus



A	Incubator on top of a heated stage
B	Zeiss Inverted Microscope
C	Micro-manipulator
D	Femtojet pump
E	CO_2 Controller Box
F	Temperature Controller Box
G	Injector Arm

2.3 Immunocytochemistry

Coverslips were removed from their wells, washed in PBS-A, and placed on pedestals (plastic tube tops) glued to plastic 90mm tissue culture dishes. Cells were fixed for 10min with 4% formaldehyde (TAAB) in cytoskeletal buffer (see 2.12). In some cases 0.2% gluteraldehyde (Sigma) was included if antibody staining permitted. For staining of intracellular proteins, cells were incubated with 0.2% Triton X-100 (Sigma) for 5min. Free formaldehyde and aldehydes were quenched by addition of 0.5mg/ml sodium borohydride (Sigma). Cells were incubated with primary antibodies for 45min-1h and binding detected by incubation with fluorescent secondary antibodies for 30-45min. F-actin in retraction fibres and stress fibres was visualised using FITC- or TRITC-conjugated phalloidin alone or included with secondary antibodies. Coverslips were mounted onto glass slides using Mowiol containing phenylenediamine as an anti-fade agent (see 2.12). Cells were washed in between each stage with PBS-A and all dilutions were carried out in PBS-A unless otherwise stated. All incubations were at room temperature. See 2.13 for details of antibodies, suppliers and dilutions.

2.3.1 Concanavalin A staining

Concanavalin A (Con A) is a lectin that binds to carbohydrate moieties on the surface of cells. Con A binding was used as an alternative to fluorescent phalloidin, to detect retraction fibres that are revealed more clearly with a membrane marker than with an F-actin marker. Cells were fixed as above, in 4% formaldehyde with 0.2% gluteraldehyde that preserves cell processes better than formaldehyde alone, in cytoskeletal buffer for 10min. This was followed by incubation with biotinylated succinylated Con A (20μg/ml; Vector Labs) for 1h. Cells were then incubated with Vectastain solution (prepared according to manufacturers instructions; Vector labs) for 30min. This solution consists of an avidin and biotinylated horseradish peroxidase macromolecular complex (ABC) that binds to biotinylated Con A, and due to the extraordinarily high affinity of avidin for biotin is essentially irreversible. Horseradish peroxidase is then visualised by the development of a peroxidase substrate (in this case DAB; 3,3'-diaminobenzidine) that produces a colour. Cells

were transferred to 4-well multiwell dishes containing at least 500µl of filtered DAB solution (prepared according to manufacturer's instructions; Vector Labs). DAB undergoes an oxidation reaction in the presence of hydrogen peroxide, producing a brown colour reaction. Cells were incubated in the dark until they turned grey-black (achieved by adding nickel chloride when the DAB solution is prepared), which was typically up to 1h, and mounted as above in Mowiol. Cells were washed with PBS-A after each stage.

2.4 Swiss 3T3 loss of cell-cell contact assay

Cells were seeded onto glass coverslips in DMEM/4%FCS as described. Post-microinjection cells were left to express ephrin-B2 or mutant constructs (see Table 2.1) for 2h before treatment with pre-clustered EphB2-Fc (R&D). Receptor bodies were pre-clustered at 37°C with anti-human IgG (R&D) at a 1:10 ratio (1µg of EphB2-Fc and 10µg of IgG per ml) for 20min before treatment. Cells were incubated with pre-clustered receptor bodies for up to 2h, fixed and stained for ephrin-B2 and F-actin. For some experiments cells were pre-treated with bFGF. In this instance, cells were serum-starved 16h prior to microinjection (2.1.2.3). 1.5h post-injection cells were treated with 20ng/ml bFGF (R&D), left for 30min and stimulated with pre-clustered EphB2-Fc (1µg/ml) as described. After stimulation, cells were fixed and stained to detect injection marker, ephrin-B2 and F-actin (see 2.3). The number of expressing cells losing contact with their neighbours within the monolayer was then analysed. A cell was scored to have lost cell-cell contact if more than 40% of the cell circumference had lost contact with the cell monolayer.

2.5 HUAEC retraction assay

Sub-confluent HUAECs grown on coverslips were removed from 60mm dishes, transferred to 4-well multiwell dishes and left for a minimum of 30min. For some experiments cells were serum-starved (16h) prior to stimulation (2.1.3.2). Cells were stimulated with pre-clustered EphB4-Fc (5µg/ml; R&D) for up to 30min. EphB4-Fc was pre-clustered with anti-human IgG (1.78µg/ml) at a 2:1 molar ratio of EphB4-Fc:IgG at 37°C for 20min prior to stimulation. Cells treated with EphB4-Fc were

fixed and stained for F-actin or Concanavalin A (see 2.3). Retracted cells were then counted. Retracted cells were defined as those displaying retraction fibres, those that had rounded and those with surface blebs The proportion of retracted cells displaying membrane blebbing was also counted separately. For some experiments cells were microinjected with mutant ephrin-B2 constructs, or other expression constructs (see Table 2.1) before EphB4-Fc stimulation.

2.6 Statistical Analysis

All error bars represent ± the standard error of the mean for that data set. Data was analysed using the software package GraphPad Instat (GraphPad Software Inc., CA, USA). Where only two data sets were compared to each other the students two-tailed T-test for unequal variance was used, represented as a *P* value. In order to test whether multiple conditions were significantly different from each other it was not appropriate to use the students T-test since multiple tests on the same data would have been necessary, which increases random error. Therefore a one-way analysis of variance (ANOVA) was performed where more than two groups were compared (result shown as an ANOVA *P* value). This allowed comparisons across groups of data and was followed by the Tukey-Kramer multiple comparisons test which was used to determine significant differences between individual groups of data, (result shown as a *P* value). *P* values of less than 0.05 were considered to be significant. Asterisks on graphs were used to denote the following: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; ns = not significant

2.7 Pharmacological inhibitors

Both the Swiss 3T3 loss of cell-cell contact assay and the HUAECs retraction assay were manipulated using a range of pharmacological inhibitors to help dissect the signalling pathways involved. Inhibitors were also used during phase time-lapse microscopy (see 2.11.1). Table 2.2 details the inhibitors used.

Table 2.2 Range of pharmacological inhibitors used

Inhibitor	Blocks	Supplier	Diluted in	Final conc.
Y27632	Rho kinase	Welfide Corp.	Water	20μM
Blebbistatin	Myosin II ATPase	Tocris	DMSO	100μM
SP600125	JNK	Tocris	DMSO	30μM
UO126	MAP kinase kinase (MEK1 & MEK2)	Tocris	DMSO	20μM
PP2	Src family kinases	Calbiochem	DMSO	10μM
SU6656	Src family kinases	Calbiochem	DMSO	5μM

2.8 Stripe assay

The stripe assay is a choice assay. It was originally developed to test the repulsive or attractive effects of axon guidance molecules such as the ephrins in the retinotectal system (Vielmetter et al., 1990; Walter et al., 1987). Cells are given the option of two substrates over which to migrate (or adhere). The substrates are laid down in stripes using specially developed silicon matrices containing flow channels; upon injection of fluid into these lanes, non-specific adsorption of proteins to the glass coverslip occurs. Where the coverslip is apposed to the matrix adsorption cannot occur. Once the coverslip is removed from the matrix, a second solution is applied to coat those areas previously hidden and create the second stripe. The first stripe formed is 50μm in width; the second stripe is 40μm wide. A variation of a published protocol (Eickholt et al., 1999) was used to prepare the stripes for HUAEC migration experiments and the protocol was developed and carried out by Iwan Evans (laboratory of Dr. Catherine Nobes). Stripes were patterned onto acid-washed glass coverslips (the protocol is summarised in Table 2.3), and HUAECs required no additional extracellular matrix adsorption. Pre-clustered EphB4-Fc (10μg/ml) was injected into the channels and the coverslip incubated at 37°C for 45min, followed by a blocking step with BSA (2mg/ml) and incubation at 37°C for 15min. A more dilute (1:5) pre-clustered Fc solution was then applied to the whole coverslip to form the second (narrower) set of stripes followed by incubation at 37°C for 1h. More dilute solutions are applied to the coverslips in an attempt to equalise adsorption in the first and second sets of stripes (smaller volumes of solution come into contact with the first set of stripes). This is followed by a second blocking step with BSA (2mg/ml) and incubation at 37°C for 15min. In order to make control stripes the pre-clustered EphB4-Fc solution is replaced with pre-clustered Fc solution. The first stripes

(50μm) were imaged via staining for the antibody clustering EphB4-Fc (Eph/Fc stripes) or Fc (Fc/Fc stripes).

Table 2.3 Method used to prepare stripes

Method	Stripes for HUAECs
Step 1 **1st injection** **(1st stripes - 50μm)**	50μl of PBS containing 10 μg/ml EphB4-Fc pre-clustered in a ratio of 2:1 of EphB4-Fc dimers:Rabbit anti Human IgG 45 min, 37°C
Step 2 (2nd injection)	100μl BSA block (2mg/ml in PBS) 15min 37°C
Step 3 (3rd injection)	100μl PBS flush
Step 4	remove from matrix
Step 5	PBS wash x2 (100μl)
Step 6 **1st Treatment** **(2nd stripes - 40μm)**	pre-clustered Fc at same molarity as 10μg/ml EphB4 - clustered with Goat anti Human IgG, then diluted 1:5 (100ul) 1h 37°C
Step 7	PBS wash x2 (100ul)
Step 8 (2nd treatment)	BSA block (2mg/ml in PBS) 15min 37°C
Step 8	PBS wash x2 (100μl) then left 30' with media in incubator before cells plated
Detection	Goat anti Rabbit cy3
Reference	modified from Eickholt et. al., 1999

HUAECs were plated onto the striped coverslips following trypsinisation as described previously and left overnight to adhere and migrate. The following day the coverslips were fixed and stained to detect the stripes, as described, and F-actin of HUAECs using FITC-conjugated phalloidin. The percentage of cells adhering to the coverslip on and off the red (50μm) stripes was then calculated.

2.9 Molecular biology techniques

2.9.1 Transformation and purification of DNA expression constructs

For bacterial transformation 1-3μl DNA was added to 50μl DH5α competent cells (Gibco), and the tube gently tapped to mix. Cells were incubated on ice for 30min, heat shocked at 37°C for 20sec and then placed on ice for 2min. 950μl SOC medium (Gibco) was added and cells incubated at 37°C with shaking for 1h. After

expression, a range of dilutions were plated onto LB agar plates containing the relevant antibiotic supplement and incubated at 37°C overnight.

5ml LB Broth with the relevant antibiotic selection was inoculated with a single transformed colony and incubated overnight at 37°C with shaking. The culture was then taken through the Qiagen mini-prep protocol as per the manufacturer's instructions to purify DNA.

In order to purify a larger quantity of plasmid DNA, 100ml LB Broth with the relevant antibiotic selection, was inoculated with 100µl of the relevant mini-prep (or starter culture if no mini-prep was done) and incubated overnight at 37°C with shaking. The culture was then taken through the Qiagen maxi-prep protocol as per the manufacturer's instructions. Constructs were verified by restriction enzyme digest and the OD at 260nm measured to give the DNA concentration.

For Rho pull downs 2µl GST-RBD DNA (Rhotekin Rho binding domain in the pGEX-2T vector; 1mg/ml stock; a kind gift from Dr. Harry Mellor) was added to 200µl pLysBL21 competent cells (a kind gift from Dr. Harry Mellor). Cells were incubated on ice for 30min, heat shocked at 42°C for 45sec and placed on ice for 2min. 0.5ml LB was added and cells incubated at 37°C for 45min with shaking. 300µl cells were plated onto an LB agar plate and a 1:10 dilution plated onto a second plate. Ampicillin (100µg/ml; Sigma) and Chloramphenicol (34µg/ml; Sigma) selection was used and the plates incubated at 37°C overnight.

2.10 Biochemical techniques

2.10.1 Cell lysis

2.10.1.1 Swiss 3T3 fibroblasts

Cells were washed in cold Tris buffered saline (TBS; see 2.12) and lysed in RIPA buffer (see 2.12) on ice. Cells were dislodged with a cell scraper (Falcon) and the

lysates were transferred to 1.5ml eppendorfs, and spun down at 13,000rpm for 10min at 4°C, to pellet any cell debris. The supernatant was transferred to a new tube and passed through a 19G needle 4 times. 20µl lysate was removed for protein estimation (carried out using the Bio-rad D_C protein assay system; as per the manufacturers instructions). An equal volume of 2 x Laemelli sample buffer (Sigma) was added to the remaining supernatant and boiled for 5min at 100°C. For the best results samples were used immediately, although it was possible to store samples at -20°C for use at a later date.

2.10.1.2 HUAECs

For ephrin-B, MYPT1 and ROCK blots, HUAECs were lysed directly into 0.5-1ml warm 1xNupage non-reducing sample buffer (Invitrogen) plus 40mM DTT (dithiothreitol; Sigma). Following scraping cell lysates were transferred to 1.5ml eppendorfs. The lysates were very viscous and were passed through a 19G needle 4 times, followed by centrifugation at 13,000rpm for 10min. The supernatant was then transferred to a new 1.5ml eppendorf and boiled for 5min at 100°C. Samples were run on SDS-PAGE gels immediately but could be stored at -20°C for use at a later date.

For Rho pull downs cells were lysed in 800µl lysis buffer (see 2.12). After cell scraping, lysates were transferred to 1.5ml eppendorfs and put through a 19G needle 4 times, then centrifuged at 13,000rpm for 10min at 4°C. Supernatants were removed to fresh tubes and a 20µl aliquot taken for a total Rho protein determination by western blotting. 40µl 1xNupage non-reducing sample buffer + 40mM DTT was added and the samples heated to 100°C for 10min before being stored on ice until loaded. The remaining 780µl was kept on ice to be used for Rho-GTP determination (see 2.10.3.3).

2.10.2 SDS-PAGE and western blotting

SDS-polyacrylamide gels (see 2.12) were poured by hand. The percentage gel varied with the molecular weight of the protein of interest: high molecular weight proteins

(such as MYPT1) required an 8% gel; mid-range molecular weight proteins, (such as ephrin-Bs) required a 10 or 12% gel; low molecular weight proteins (such as Rho) required a 15% gel. Proteins were run in running buffer (see 2.12) in the Mini-PROTEAN 3 system (Bio-Rad). Typically protein was run through the stacking portion of the gel at 100V and the voltage then increased to 150V for 1-1.5h until an adequate separation of rainbow markers (Amersham) was seen. Protein was then transferred to a methanol-pre-treated PVDF membrane (Amersham) at 100V for 2h in transfer buffer (see 2.12) using Mini Trans-Blot Electrophoretic Transfer Cells (Bio-Rad).

PVDF membranes were blocked overnight at 4°C (or 1h at room temperature) in Superblock (Perbio). 5% BSA (Sigma) was used as a blocking agent specifically when detecting phosphoMYPT1 Thr850 and Thr696. Primary antibodies were diluted in TBS-Tween (see 2.12) with Superblock or 5% BSA added at a dilution of 1:5. The filter was incubated for 60-90min at room temperature in primary antibody and washed in TBS-Tween (3x15min washes). HRP-conjugated secondary antibodies, diluted in TBS-Tween with Superblock or 5% BSA added at a dilution of 1:10, were then applied for 45min at room temperature. After 3x15min TBS-Tween washes the protein bands were revealed using SuperSignal West Pico Chemiluminescent Substrate (Pierce) to detect bound HRP. Luminescence was detected using CL-Xposurefilm (Pierce). For details of antibodies and dilutions see 2.13.

PVDF membrane could be stripped to enable re-probing. This involved 2x25min washes with ImmunoPure IgG elution buffer (Pierce). Following stripping the filter must be washed in TBS-Tween and re-blocked as described above before re-probing can be done.

2.10.3 Rho GTPase pull down assay

2.10.3.1 Purification of GST-RBD

100ml LB Broth (100μg/ml Ampicillin; 34μg/ml Chloramphenicol) was inoculated with a single colony of GST-RBD and incubated overnight at 37°C with shaking. The bacterial culture was then diluted 1:20 in fresh LB Broth (100μg/ml Ampicillin) and grown to OD600=0.7 (about 1.75h). Protein expression was induced with IPTG (isopropyl-β-D-thiogalactopyranoside; Calbiochem) at a final concentration of 0.5mM and the culture incubated for a further 2h at 30°C with shaking. Cultures were collected by centrifugation at 3000rpm in 50ml tubes (Falcon), the supernatant was discarded and the pellets stored at -80°C until the next phase of the assay was undertaken.

Pellets from 300ml (6 x 50ml falcons) of culture were used per experiment, enough for 8 samples. All six pellets were resuspended in 4.5ml ice-cold STE buffer (see 2.12) with 1mM PMSF (phenylmethylsulphonyl fluoride; Amersham) and homogenised with a 19G needle (~8 passes). 100μg/ml lysozyme (Sigma), dissolved in 500μl STE buffer prior to use, was added and the suspension mixed (rocked by hand 6-7 times), and incubated on ice for 15min. 5mM DTT was then added and the suspension mixed (rocked by hand 6-7 times). Tween-20 (Amersham) to a final concentration of 1% and SDS (Sigma) to a final concentration of 0.03% were then added and the suspension mixed (rocked by hand 8-10 times). The suspension was aliquoted into 1.5ml eppendorfs and centrifuged at 14,000rpm for 30min at 4°C. Supernatant was transferred to a clean 15ml tube and 150μl prewashed (with cold STE buffer) glutathione sepharose beads (Amersham) were added to bind GST-RBD, followed by incubation for 10min at room temperature with gentle rocking. The suspension was then centrifuged at 2000rpm for 3min at 4°C to pellet the beads. Beads were washed with ice-cold STE buffer 3 times (spun at 2000rpm for 2min at 4°C), and resuspended in 120μl STE buffer (total volume about 200μl). For determination of protein loading on beads, 10μl of beads were removed and an equal volume of 1 x Nupage non-reducing sample buffer + 40mM DTT added. This was used to estimate the amount of protein loaded on the beads by coomassie staining

(see 2.10.3.2). The bead preparation was stored on ice until cell lysates were prepared (typically 1h).

2.10.3.2 Coomassie staining for protein

Samples of bacterial culture pre- and post-IPTG induction, together with a sample of the GST-RBD bead preparation and BSA protein standards, were run on a 15% SDS-PAGE gel for 1h 15min at 150V. The gel was then incubated with 15ml filtered coomassie stain (see 2.12). Incubation ranged from a couple of hours to overnight with rocking. After staining the gel was washed in coomassie de-stain (see 2.12) until all protein bands were clearly visible. The gel could now be dried in a gel drying system (Sigma). The gel was incubated in equilibration buffer (see 2.12) for a minimum of 30min with rocking, placed between two sheets of cellophane in the gel drying apparatus (Sigma) and left overnight until free of moisture. The amount of protein on the beads and the extent of IPTG induction were estimated by comparing to the known BSA standards (see Fig. 5.6).

2.10.3.3 Rho Pull down

In order to determine changes in the amount of cellular Rho-GTP during pre-clustered EphB4-Fc induced retraction of HUAECs the Rho pull down assay was used (method modified from Ren and Schwartz, 2000). Approximately 25µl (30-40µg) GST-RBD beads were added to each cleared lysate and samples rotated at 4°C for 45 min. The beads were then washed four times (in a microfuge at 5000rpm for 20sec at 4°C) with 600µl cold Tris buffer (see 2.12). After the last wash the Tris buffer was removed carefully, 30µl hot 1 x Nupage non-reducing sample buffer + 40mM DTT added to elute and all samples heated to 100°C for 10min. The entire volume of Rho-GTP beads was loaded (samples were spun briefly before loading to ensure accuracy). Samples were then run alongside a 15µl aliquot of the 20µl sample mixed with sample buffer taken prior to the pull down (see 2.10.1.2). This was loaded as a measure of total Rho levels, (although volumes of lysates were measured after lysis and loading adjusted accordingly), on a 15% SDS-PAGE gel as described in 2.10.2. For antibody details see 2.13.

2.11 Image acquisition

2.11.1 Phase time-lapse microscopy

A coverslip of sub-confluent HUAECs was placed in a 35mm glass-bottomed dish (World Precision Instruments) containing 1.5ml full medium ± inhibitors (such as Y27632 or SP600125) on the heated stage of an inverted microscope (Axiovert 200M; Zeiss) and viewed using either 20x or 40x objectives. Cells were stimulated by the addition of 500µl medium containing appropriate stimulant to the dish. Cells were maintained at 37°C, 5% CO_2 throughout. Time-lapse images were collected using a Hamamatsu camera and Openlab software (Improvision). Movies were collected one frame every 15sec and generally recorded over a period of 1h.

2.11.2 Confocal microscopy

Fluorescent images were obtained using either a Leica upright microscope or a Leica DMIRE2 inverted microscope, and images acquired using a Leica AOBS SP2 confocal imaging system. Images were taken with the 40x and 60x oil immersion objectives. Lasers used: Argon for FITC; Krypton-Argon for TRITC, Cy3 and Texas Red; Ultra violet for Alexa-350. Images were processed using the Leica confocal software and Adobe Photoshop.

2.11.3 Wide-field microscopy

Images were acquired using a Zeiss Axioplan II microscope with a Hamamatsu (ORCA-ER) camera. Oil immersion 40X Plan-Neofluar (NA 1,3) objective or an oil immersion 63X Plan Neofluar (NA 1,25) objective were used. For the stripes however a 10X Acroplan (NA0,25) air objective was used.

2.12 Media and Solutions

Serum Free Medium

DMEM Powder	3.34g
Sodium Bicarbonate	0.5g
Pen/Strep	2.5ml
Sodium Pyruvate	2.5ml

Make up to 250ml with distilled water and filter sterilise.

Store in incubator at 37°C with the lid loose.

Cytoskeletal Buffer

MES (pH 6.1)	10mM
KCl	125mM
MgCl	2mM
EGTA	2mM
Sucrose	10%

For Fix:

Formaldehyde	4%
Gluteraldehyde	0.2% (optional)

Mowiol

Glycerol	25%
Mowiol	10%
Tris HCL pH 8.5-8.8	0.1M
Phenylenediamine	2 crystals per 5ml aliquot

Store in 50µl aliquots at -80°C

Light sensitive

RIPA Buffer (Lysis buffer)

Tris, pH 7.4	20mM
NaCl	125mM
Triton X-100	1%
Sodium Deoxycholate	0.5%
SDS	0.1%
Glycerol	10%
EDTA	5mM
NaF	50mM
$NaVO_3$	0.5mM
Complete Protease Inhibitor tablet	1 tablet per 100ml
PMSF*	100µg/ml

Make up fresh for every experiment.

* Add immediately prior to lysis.

TBS

Tris pH 7.4	20mM
NaCl	150mM

Make up to 1L with water

Store at 4°C

TBS-Tween

20mM Tris pH 7.4	20mM
150mM NaCl	150mM
Tween 20	0.1%

Make up to 1L with water

Store at 4°C

Polyacrylamide Gels

Resolving gel (15ml)

	8%	10%	12%	15%
30% acrylamide mix	4000ul	5000ul	6000ul	7500ul
1.5M Tris pH 8.8	3800ul	3800ul	3800ul	3800ul
10% SDS	150ul	150ul	150ul	150ul
10 % APS	150ul	150ul	150ul	150ul
TEMED	9ul	6ul	6ul	6ul
UP Water	6900ul	5900ul	4900ul	3400ul

Stacking gel (4ml)

30% acrylamide mix	670ul
1.5M Tris pH 6.8	500ul
10% SDS	40ul
10 % APS	40ul
TEMED	4ul
UP Water	2700ul

Running Buffer (10x)

250mM Tris	250mM
1.92M Glycine	1.92M
1% SDS	1%

Make up to 1L in Water

For 1x dilute 1 in 10 in water

Transfer Buffer (10x)

480mM Tris	480mM
390mM Glycine	390mM
0.37% SDS	0.37%

Make up to 1L in water

For 1x use 100ml 10x plus 200ml methanol plus 700ml water

Coomassie Stain

Methanol	40%
Acetic acid	7%
Coomassie Blue	0.25%
Distilled Water	52.75%

Coomassie De-stain

Methanol	10%
Acetic Acid	10%
Distilled Water	80%

Equilibration Buffer

Ethanol	20%
Glycerol	10%
Distilled Water	70%

STE Buffer:

Tris, Ph 8.0	10mM
NaCl	150mM
EDTA	1mM

Make up in distilled water

Lysis Buffer (Rho Pull Down)

Tris pH 7.2	50mM
Triton X-100	1%
SDS	0.1%
NaCl	500mM
MgCl2	10mM
Aprotinin	10ug/ml
Leupeptin	10ug/ml
PMSF	1mM

Make up in distilled water

Tris Buffer (Rho Pull Down)

Tris pH 7.2	50mM
Triton X-100	1%
NaCl	150mM
MgCl2	10mM
Aprotinin	10ug/ml
Leupeptin	10ug/ml
PMSF	1mM

Make up in distilled water

2.13 Antibodies

2.13.1 Primary Antibodies

Primary antibody	Species	Supplier	ICC* dilution	WB* dilution
ephrin-B1	Rabbit	Zymed	1:100	1:1000
ephrin-B2	Goat	R & D	1:150	1:1000
ephrin-B2	Rabbit	Aviva	1:100	1:1500
ephrin-B3	Rabbit	Zymed	1:100	1:1000
Myosin Phosphatase	Rabbit	Covance	N/A	1:2000
Phospho MYPT1 Thr 696	Rabbit	Upstate	N/A	1:1500
Phospho MYPT1 Thr 850	Rabbit	Upstate	N/A	1:1500
RhoA	Mouse	Santa Cruz	N/A	1:1000
ROCK I	Mouse	BD Transduction Labs	N/A	1:250
ROCK II	Mouse	BD Transduction Labs	N/A	1:1000
ß-catenin	Mouse	BD Transduction Labs	1:100	N/A
Myc	Mouse	Serotec	1:200	N/A
Paxillin	Mouse	Zymed	1:50	N/A

N.B. EphB-Fcs were used as primary antibodies to ephrin-B ligands in fixed cells at a dilution of 1:100 (stock = 200ug/ml)

* ICC = Immunocytochemistry
WB = Western Blot

2.13.2 Secondary Antibodies

species	antibody	fluorophore	supplier	diluted in	dilution
donkey	anti goat	Cy3	Stratech	PBS-A	1:300
donkey	anti goat	FITC	Stratech	PBS-A	1:200
donkey	anti goat	Texas Red	Stratech	PBS-A	1:200
donkey	anti human	FITC	Stratech	PBS-A	1:200
goat	anti human	Cy3	Stratech	PBS-A	1:300
donkey	anti mouse	FITC	Stratech	PBS-A	1:200
donkey	anti mouse	Cy3	Stratech	PBS-A	1:200
goat	anti mouse	FITC	Stratech	PBS-A	1:200
donkey	anti mouse	FITC	Stratech	PBS-A	1:200
donkey	anti rabbit	FITC	Stratech	PBS-A	1:200
donkey	anti rabbit	Texas Red	Stratech	PBS-A	1:200
donkey	anti rabbit	TRITC	Stratech	PBS-A	1:200
donkey	anti rabbit	HRP	Stratech	PBS-A	1:30,000
donkey	anti goat	HRP	Stratech	PBS-A	1:30,000
goat	anti mouse	HRP	Stratech	PBS-A	1:30,000
streptavidin	(binds biotin)	Alexa350	Molecular Probes	PBS-A	1:200
phalloidin	(binds F-actin)	FITC	Sigma	PBS-A	1:200
phalloidin	(binds F-actin)	TRITC	Sigma	PBS-A	1:500
DAPI	(binds DNA)	/	Sigma	Water	1:10,000

Chapter 3

Clustering of ephrin-B2 expressed in Swiss 3T3 fibroblasts causes loss of cell-cell contact

3.1 Introduction

Eph receptor/ephrin signalling has been shown to mediate both repulsive and attractive cell migrations in developing and adult tissues, and due to the membrane bound nature of both receptors and ligands, Eph receptor/ephrin activation requires direct cell-cell contact between receptor and ligand expressing cells. Signalling is bi-directional; both Eph receptors and ephrin ligands are capable of sending and receiving a signal.

Eph/ephrin signalling has been shown to influence many developmental processes including neural crest cell migration, tissue boundary formation, axon guidance and angiogenesis. For example, segmental expression of ephrins in the somites is thought to guide early trunk neural crest cell migration (Krull et al., 1997; Wang and Anderson, 1997). In addition, Eph/ephrin signalling is responsible for the restriction of cell intermingling during hindbrain segmentation (Mellitzer et al., 1999; Xu et al., 1999), and, more recently, has been implicated in the migration and adhesion of cells involved in the septation of the urorectal region (Dravis et al., 2004). Signals mediated by Eph receptors and ephrins also plays many roles throughout axon guidance. One of the most widely used models to study this is the projection of retinal axons to the midbrain. Eph receptors and ephrins have been shown to function as guidance molecules, vital for correct topographic mapping to occur (reviewed in Flanagan and Vanderhaeghen, 1998; Huber et al., 2003; O'Leary and Wilkinson, 1999). Furthermore, Eph receptors and ephrins also function to regulate blood vessel development throughout angiogenesis (reviewed in Adams and Klein, 2000; Cheng et al., 2002a). For example, EphB4 and ephrin-B2 play a role during the specification of arteries and veins (Wang et al., 1998). Ephrin-B signalling is known to regulate the actin cytoskeleton and cell adhesion (Cowan and Henkemeyer, 2001; Huynh-Do et al., 2002; Xu et al., 2003), but the signalling pathways involved have not yet been fully characterised.

Ephrin-B ligands become phosphorylated on tyrosine upon stimulation with soluble pre-clustered EphB-Fc fusion proteins (Bruckner et al., 1997; Holland et al., 1996) and co-culturing cells expressing ephrin-Bs with EphB receptor expressing cells

results in tyrosine phosphorylation of both receptors and ligands (Holland et al., 1996). Ephrins do not possess a catalytic domain, but tyrosine phosphorylation of ephrin-Bs has been shown to occur via Src family kinases (Bruckner et al., 1997; Holland et al., 1996; Palmer et al., 2002), and is necessary for some reverse signalling events. For example, sprouting angiogenesis of endothelial cells triggered by EphB4-Fc is inhibited upon treatment with the Src family kinase inhibitor PP2 (Palmer et al., 2002). In addition, other receptor tyrosine kinase pathways have been implicated in ephrin-B activation. Platelet-derived growth factor (PDGF) stimulation of ephrin-B expressing cells can result in tyrosine phosphorylation of the ligand (Bruckner et al., 1997; Palmer et al., 2002), and serum-triggered phosphorylation of ephrin-Bs in endothelial cells has been shown to be unaffected by inhibitors of Src family kinases, indicating other kinases are responsible for this induction (Palmer et al., 2002). The Tie-2 receptor tyrosine kinase has also been shown to phosphorylate ephrin-B2 *in vitro* (Adams et al., 1999). In addition, activated fibroblast growth factor receptor (FGFR) can induce tyrosine phosphorylation of endogenous ephrin-Bs in chick primary retina *in vivo* (Chong et al., 2000), and expressed ephrin-B1 in *Xenopus* oocytes (Bong et al., 2004).

Ephrin-B proteins have well-conserved cytoplasmic domains, particularly the C-terminal 33 amino acids, which show nearly 100% identity among the three known ephrin-B ligands. Within these last 33 amino acids are five tyrosine residues, and a potential PDZ binding motif (Song et al., 2002). In avian ephrin-B1, tyrosine residues at positions 312, 317 and 331 (corresponding to 311, 316 and 330 in mouse) are phosphorylated upon Eph receptor engagement *in vivo* (Kalo et al., 2001). Tyr-331 is within the PDZ binding domain at the C-terminus of ephrin-Bs and has been shown to be the major *in vivo* phosphorylation site of ephrin-B1 in the ephrin-B1/EphB2 complex. It is postulated that this residue plays a role in the multimerisation of ephrin-B molecules (Kalo et al., 2001). Interestingly, it has recently been shown that murine Tyr-304 is crucial for Grb4 binding via its SH2 domain (Su et al., 2004), as is the equivalent residue (Tyr-298) in *Xenopus* (Bong et al., 2004). This residue is not reported as an *in vivo* phosphorylation site, but it is possible that phosphorylation of Tyr at positions 311 and 316 could induce a conformational change that positions Tyr-304 to interact with Grb4 (Su et al., 2004).

Ephrin-B signalling is not entirely phosphorylation dependent. Various signalling molecules have been shown to bind via the C-terminal PDZ binding motif present in all known ephrin-Bs. These include the adaptor proteins GRIP-1, GRIP-2 and syntenin, the protein kinase C-interacting protein Pick1, the protein tyrosine phosphatase PTP-BL and the GTPase activating protein (GAP) for heterotrimeric G-proteins, PDZ-RGS3 (Bruckner et al., 1999; Lin et al., 1999; Lu et al., 2001; Palmer et al., 2002; Torres et al., 1998). The significance of many of these interactions is not clear. However, PDZ proteins have been widely implicated in forming submembrane scaffolds that cluster molecules at the cell surface (Craven and Bredt, 1998; Garner et al., 2000; Sheng and Pak, 2000). Interaction between ephrin-B1 and PDZ-RGS3 was found to be necessary for ephrin-B1 mediated cell dissociation in the *Xenopus* embryo (Lu et al., 2001), and activation of reverse signalling inhibited the chemoattraction of cerebellar granule cells induced by the chemokine SDF-1. This inhibition was found to depend on cross-talk between ephrin-B1 signalling and G-protein coupled receptor signalling via PDZ-RGS3 (Lu et al., 2001). Binding of PTP-BL (a protein tyrosine phosphatase) to ephrin-B1, with delayed kinetics via its PDZ domain, results in ephrin-B1 dephosphorylation and phosphotyrosine dependent signalling is subsequently switched off (Palmer et al., 2002). Ephrin-B signalling is also responsible for a dramatic cell rounding response in HEK 293 cells which requires the MAP kinase JNK (c-jun amino terminal kinase) and is phosphorylation independent (Xu et al., 2003).

Regulation of the actin cytoskeleton is essential for guiding cell movements. Cell surface receptors can transmit signals in response to external cues that give rise to actin cytoskeletal changes, leading to the regulation of cell adhesion and motility (Cowan and Henkemeyer, 2001; Huynh-Do et al., 2002; Xu et al., 2003). Ephrin-Bs have been linked to downstream signalling partners involved in the regulation of cytoskeletal dynamics, cell migration and adhesion events. The SH2/SH3 domain protein Grb4 binds to ephrin-B1 via its SH2 domain in a phosphotyrosine dependent manner and, through its SH3 domains, links ephrin-B1 to many proteins with roles in cytoskeletal regulation (Cowan and Henkemeyer, 2001). In response to ephrin-B1 activation, a redistribution of paxillin away from focal adhesions to the cytoplasm occurs, as well as increased phosphorylation of focal adhesion kinase (FAK) at Tyr397 (Cowan and Henkemeyer, 2001). In addition, the recruitment of many other

downstream proteins via the SH3 domains of Grb4 is possible (Cowan and Henkemeyer, 2001), such as cyclase associated protein (CAP), which has been proposed to regulate actin stress fibres and focal adhesions (Ribon et al., 1998), and hnRNPK which has been shown to interact with Vav, an exchange factor for Rac (Bustelo et al., 1995; Hobert et al., 1994).

In order to understand how ephrins and Eph receptors are involved in cell migration, it is necessary to understand the cellular responses that are triggered upon ephrin activation by Eph receptors, and the signalling pathways that underlie these responses. Swiss 3T3 cells are a fibroblast cell line that has been used extensively for the investigation of actin cytoskeletal rearrangements triggered by growth factor receptors (Rankin and Rozengurt, 1994; Ridley, 1995; Ridley and Hall, 1992; Seufferlein and Rozengurt, 1994). Swiss 3T3 fibroblasts have been well characterised and are flat well–spread cells in culture, therefore an ideal cell type for microinjection.

A reasonable amount of information is known about the biochemical 'forward' signals transduced by Eph receptors. However, little is known about the signalling pathways and events triggered by ephrin-B 'reverse' signalling. It has previously been shown that BHK (Baby Hamster Kidney) fibroblast cells transfected with ephrin-B1 show a loss of actin stress fibres and cell rounding when stimulated with EphB2 (Cowan and Henkemeyer, 2001). In order to look at the cytoskeletal changes that occur upon activation of another ephrin ligand, ephrin-B2, I expressed ephrin-B2 in confluent, quiescent Swiss 3T3 fibroblasts. The downstream signalling events triggered after ephrin-B2 clustering by soluble Eph receptors were then analysed.

In this chapter, I have shown that stimulation of Swiss 3T3 cells, exogenously expressing ephrin-B2, with soluble pre-clustered EphB2-Fc triggers a loss of cell-cell contact, resulting in the ephrin-B2 expressing cells withdrawing from their neighbours within the cell monolayer. I have shown that this response requires the presence of serum factors or fibroblast growth factor (FGF). A truncated form of ephrin-B2, missing the entire cytoplasmic domain, does not trigger loss of cell-cell contact, demonstrating a dependence on the intracellular domain of the ligand. In addition, I have shown that Src family kinases, and the SH2/SH3 domain adaptor

protein Grb4, are critical for loss of cell-cell contact to occur. However, PDZ domain protein interactions are not necessary and the response is not sensitive to pre-treatment with the ROCK inhibitor, Y27632, or blebbistatin, an inhibitor of myosin II ATPase.

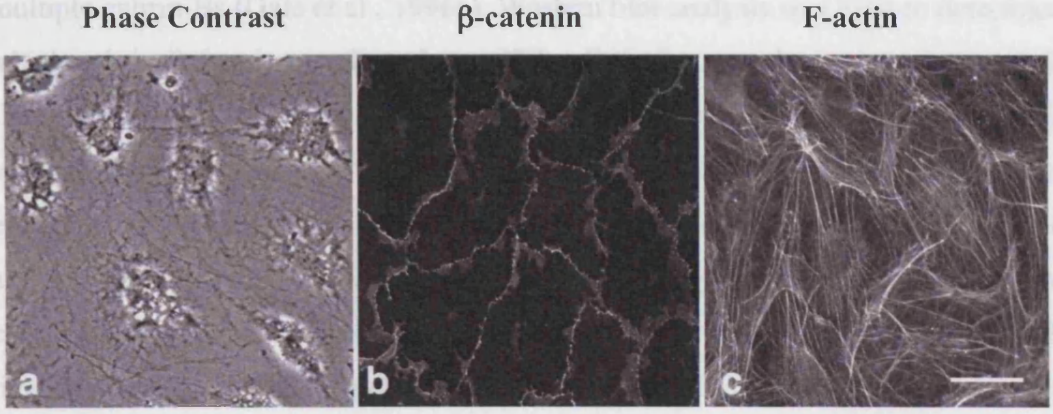
3.2 Results

3.2.1 Characterisation of Swiss 3T3 Fibroblasts

Confluent Swiss 3T3 fibroblasts were prepared as described in chapter 2. When plated at high density and grown to confluency, without medium change, cells become quiescent after 7-10 days in culture. Cells within the monolayer are closely juxtaposed (Fig. 3.1a) and have adherens junctions between cells as shown by β-catenin staining (Fig. 3.1b). The cells are flat, well-spread and without serum-starvation display some actin stress fibres (Fig. 3.1c).

Endogenous ephrin-B expression in Swiss 3T3 fibroblasts can be demonstrated by staining cells using an "Eph receptor body" (Gale and Yancopoulos, 1997), a chimeric protein composed of the extracellular ligand-binding domain of an Eph receptor, in this case EphB2, which has selective binding affinity for ephrin-Bs, fused to the Fc fragment of the heavy chain of human IgG (EphB2-Fc). Generally, EphB receptors bind ephrin-B ligands and EphA receptors bind ephrin-A ligands, and binding within subclasses is fairly promiscuous (Davis et al., 1994; Gale et al., 1996b; Gale and Yancopoulos, 1997). Binding of EphB2-Fc (pre-clustered with anti-human IgG; 20min stimulation) to endogenous ephrin-Bs was visualised after fixation with a fluorescently tagged secondary antibody. Clusters of ephrin-B are revealed evenly distributed across the fibroblast, extending to the edge of the lamellae (Fig 3.2a & red in c). Cells fixed prior to EphB2-Fc treatment, display a diffuse, rather than puncate, ephrin-B stain (data not shown), indicating that stimulation of ephrin-Bs in these cells triggers clustering of endogenous ligands. Panels d-f are controls for non-specific antibody binding; cells were treated with the isolated Fc portion of human IgG pre-clustered with anti-human IgG, and show no staining pattern (Fig. 3.2d).

Figure 3.1 Quiescent Swiss 3T3 Fibroblasts



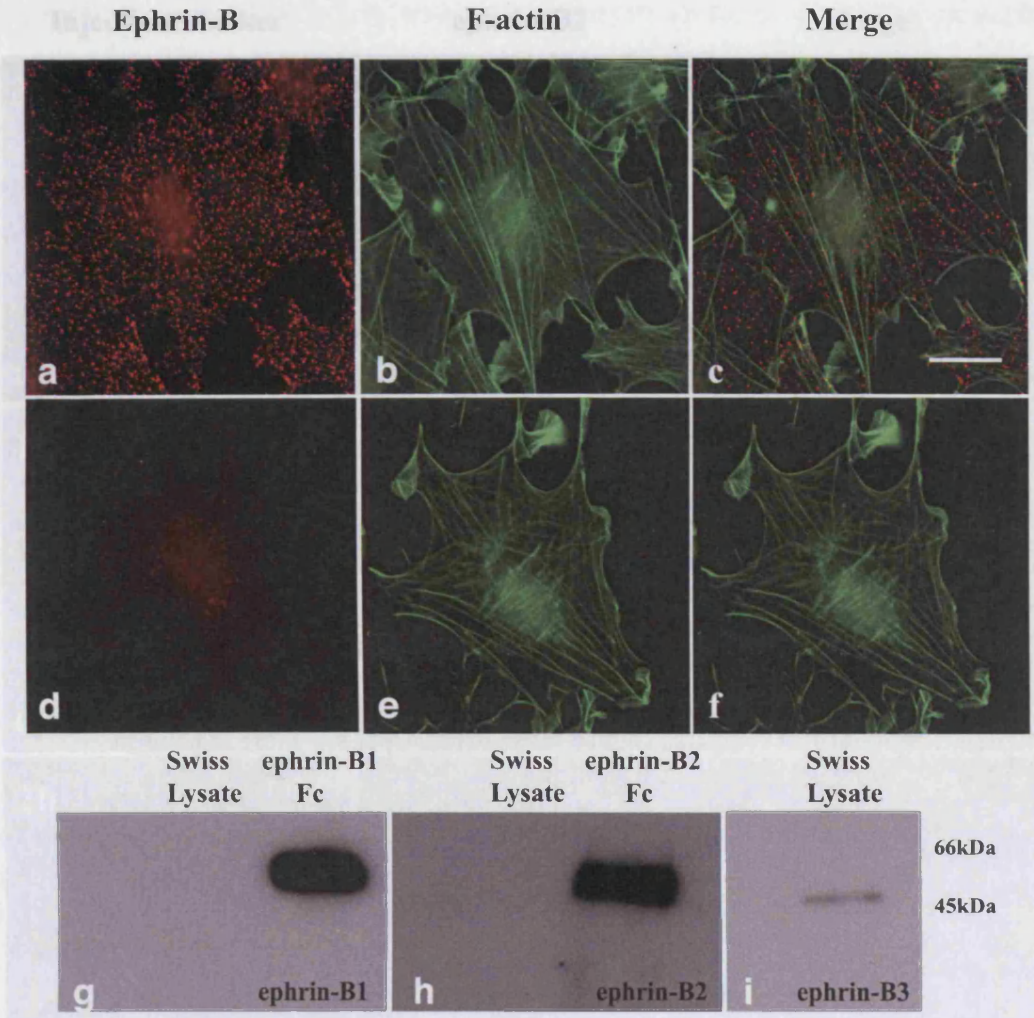
Quiescent Swiss 3T3 fibroblasts are well spread and form a monolayer as shown by phase contrast microscopy (a). The cells have adherens junctions, illustrated by β-catenin staining using an anti-β-catenin antibody (b). Phalloidin staining of filamentous actin (F-actin) reveals that these cells, without serum-starvation, display some actin stress fibres (c). Scale bar 20μm.

Immunocytochemistry using an "Eph receptor body" does not distinguish between the 3 ephrin-B family members due to the promiscuous binding of EphB receptors to multiple ephrin-Bs (Gale et al., 1996b). Western blot analysis was used to determine which ephrin-B family members Swiss 3T3 cells endogenously express. Figure 3.2i shows that Swiss 3T3 cells express ephrin-B3 (molecular weight of ephrin-Bs is approximately 45kDa (Bruckner et al., 1999; Lin et al., 1999). No expression of ephrin-B1 or ephrin-B2 was detected (Fig. 3.2g&h), although mouse ephrin-B1-Fc and mouse ephrin-B2 Fc were detected using these antibodies. Ephrin-B-Fcs are chimeric proteins composed of the extracellular domain of one of the three ephrin-B ligands fused to the Fc fragment of the heavy chain of human IgG and have a calculated molecular weight of 49.2 kDa but as a result of glycosylation the recombinant protein migrates at 60 kDa under reducing conditions. I have demonstrated using western blotting that Swiss 3T3 fibroblasts endogenously express ephrin-B3 but not ephrin-B1 or ephrin-B2.

3.2.2 Detection of expressed ephrin-B2 in Swiss 3T3 fibroblasts

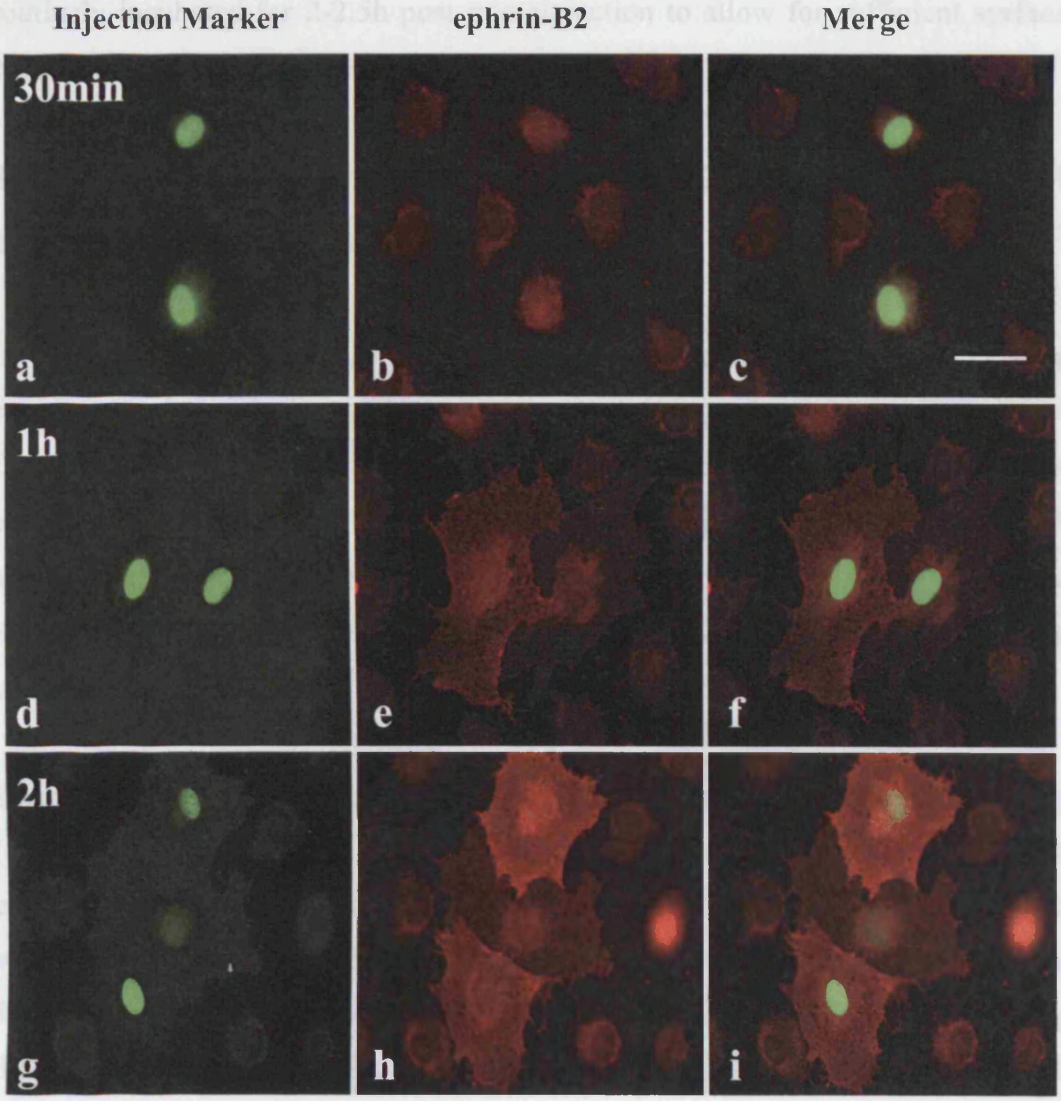
Stimulation of confluent Swiss 3T3 fibroblasts with EphB2-Fc resulted in no detectable changes to the actin cytoskeleton or cell morphology (results not shown). The levels of endogenous ephrin-B3 expression may not be sufficient to elicit a morphological response upon stimulation. Alternatively, ephrin-B3 may not trigger a morphological/actin cytoskeletal response, unlike ephrin-B1 (Cowan and Henkemeyer, 2001). These cells therefore present an ideal model system for looking at changes to the actin cytoskeleton and cell morphology triggered by activation of exogenous ephrin-B2. Ephrin-B2 was expressed by microinjection of pRK5-ephrin-B2 (200μg/ml), plus FITC dextran (5mg/ml) as an injection marker. Post-injection cells were incubated at 37°C/10%CO_2 for various times to allow for surface expression of ephrin-B2, after which they were fixed and stained, without permeabilisation, for surface ephrin-B2 using a commercially available antibody. 30min post-injection nuclear FITC dextran is clearly visible indicating injected cells (Fig. 3.3a & green in c), but no surface ephrin-B2 expression was detected (Fig. 3.3b). 1h post-injection, injected cells are again clearly identified (Fig. 3.3d & green in f), together with a low level of surface ephrin-B2 staining (Fig. 3.3e & red in f).

Figure 3.2 Endogenous ephrin-B expression in Swiss 3T3 fibroblasts



Cells were stimulated for 20min with pre-clustered EphB2-Fc and stained for F-actin with FITC-phallodin (green) and ephrin-Bs, (by detecting anti-human IgG used to cluster the bound "Eph receptor body"), with a Cy3 conjugated secondary antibody (red). Swiss 3T3 cells endogenously express ephrin-Bs (a & red in c). No staining was seen upon treatment of cells with pre-clustered Fc alone (d & f). Western blotting analysis using commercially available antibodies to the individual ephrin-Bs revealed endogenous expression of only ephrin-B3, which has a molecular weight of approximately 45kDa (i). No expression of ephrin-B1 or ephrin-B2 was detected, although the antibodies do recognise mouse ephrin-B1 Fc and mouse ephrin-B2-Fc (g&h), which have a calculated molecular weight of 49.2kDa but as a result of glycosylation migrate at 60kDa under reducing conditions. Scale bar 10μm.

Figure 3.3 Surface expression of ephrin-B2 post-microinjection



Swiss 3T3 fibroblasts were microinjected with pRK5-ephrin-B2 (200μg/ml) and incubated at 37°C/10% CO_2 for various times to allow for protein expression. FITC dextran (5mg/ml) was co-injected as an injection marker (green). Cells were fixed and not permeabilised so that only surface ephrin-B2 was detected using an anti-ephrin-B2 antibody (red). Surface ephrin-B2 expression was not detected 30min post-injection (a-c). 1h after injection a weak signal was detected in many, but not all, injected cells (d-f). After 2h all injected cells displayed a uniform diffuse ephrin-B2 stain (g-i). Scale bar 20μm.

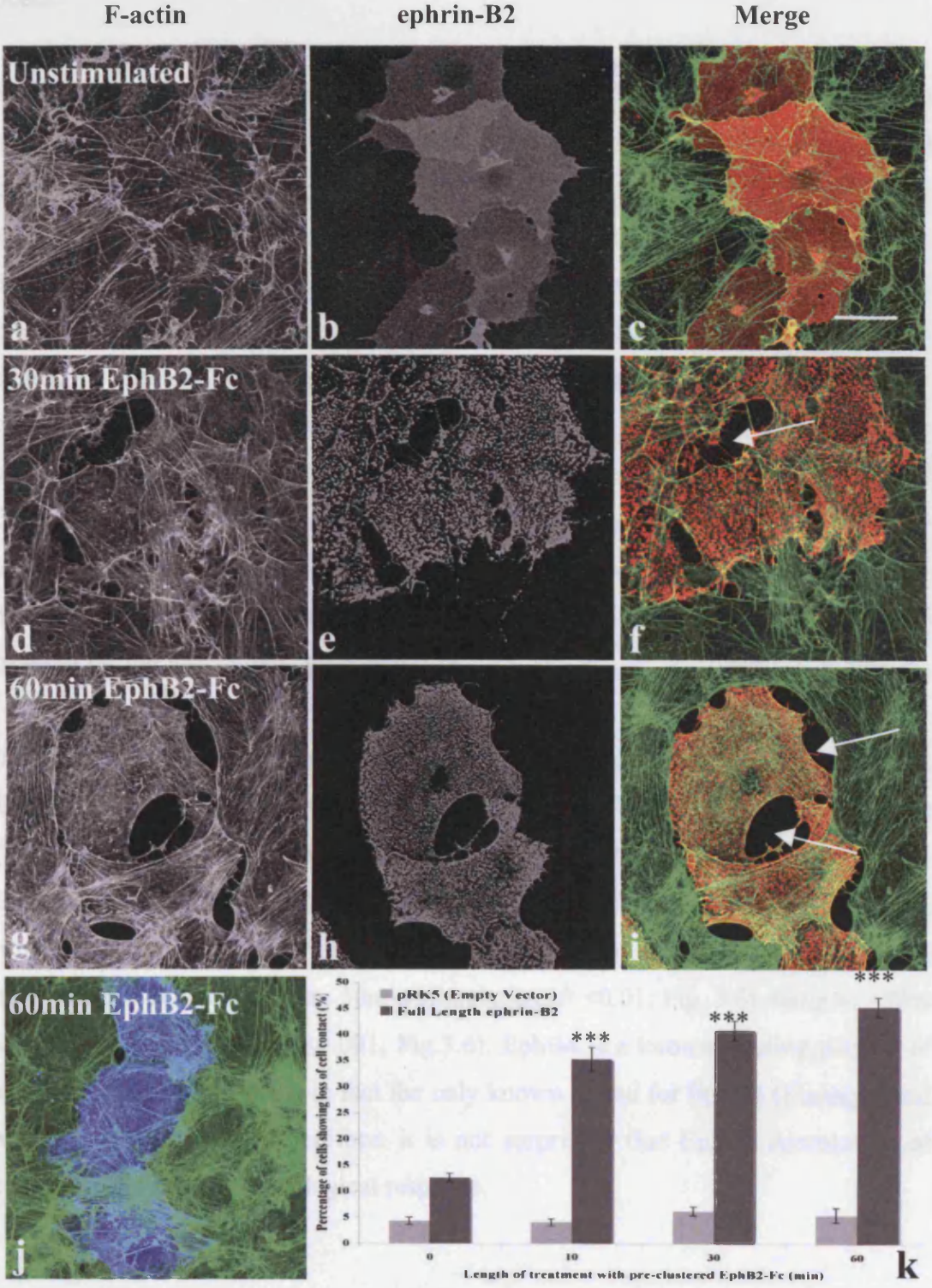
After 2h, the injected cells (Fig. 3.3g & green in i) clearly show an elevated diffuse surface expression of ephrin-B2 (Fig. 3.3h & red in i). Therefore, cells were routinely incubated for 2-2.5h post-microinjection to allow for sufficient surface expression of ephrin-B2 constructs.

3.2.3 EphB receptor stimulation of ephrin-B2 induces clustering of ephrin-B2 and loss of cell-cell contact

In order to investigate the effect of EphB2-Fc stimulation on cells expressing exogenous ephrin-B2, Swiss 3T3 fibroblasts were microinjected with pRK5-ephrin-B2 (200μg/ml) plus biotin dextran (2mg/ml) and left for 2h to express. To activate ephrin-B2 signalling, cells were exposed to 1μg/ml EphB2-Fc pre-clustered with anti-human IgG at a ratio of 1μg:10μg. Ephrin-B2 expressing cells were stimulated for between 10 and 60min after which time the cells were fixed and stained to visualise actin filaments and ephrin-B2 expression.

Unstimulated ephrin-B2 expressing cells show uniform diffuse ephrin-B2 staining (Fig. 3.4b and red in c), and display no loss of cell-cell contact compared with expression of empty vector alone (Fig. 3.4k). Ephrin-B2 expressing cells stimulated with pre-clustered EphB2-Fc initially show clustering of ephrin-B2 into spots at 10min (data not shown), and larger patches at later times (30 and 60min; Fig. 3.4e&h; red in f&i). Clustering of ephrin-B2 causes expressing cells to lose cell-cell contact and withdraw from their neighbours (Fig. 3.4d-g; arrows in f&i). Loss of cell-cell contact was defined as an expressing cell where more than 40% of its circumference had lost contact with the surrounding monolayer. A rise in the number of ephrin-B2 expressing cells exhibiting loss of cell-cell contact was observed after 10min pre-clustered EphB2-Fc stimulation ($P < 0.001$; Fig 3.4k) and increased over time, a higher proportion of cells responding after 60min stimulation compared with 10min ($P < 0.01$; Fig. 3.4k). Injection of empty vector alone prior to pre-clustered EphB2-Fc stimulation does not cause the injected cells to lose cell-cell contact (Fig. 3.4j&k) indicating the response is specific to ephrin-B2 clustering. Notably, every ephrin-B2 expressing cell does not lose cell-cell contact as a consequence of

Figure 3.4 Clustering of ephrin-B2 in Swiss 3T3 fibroblasts triggers loss of cell-cell contact

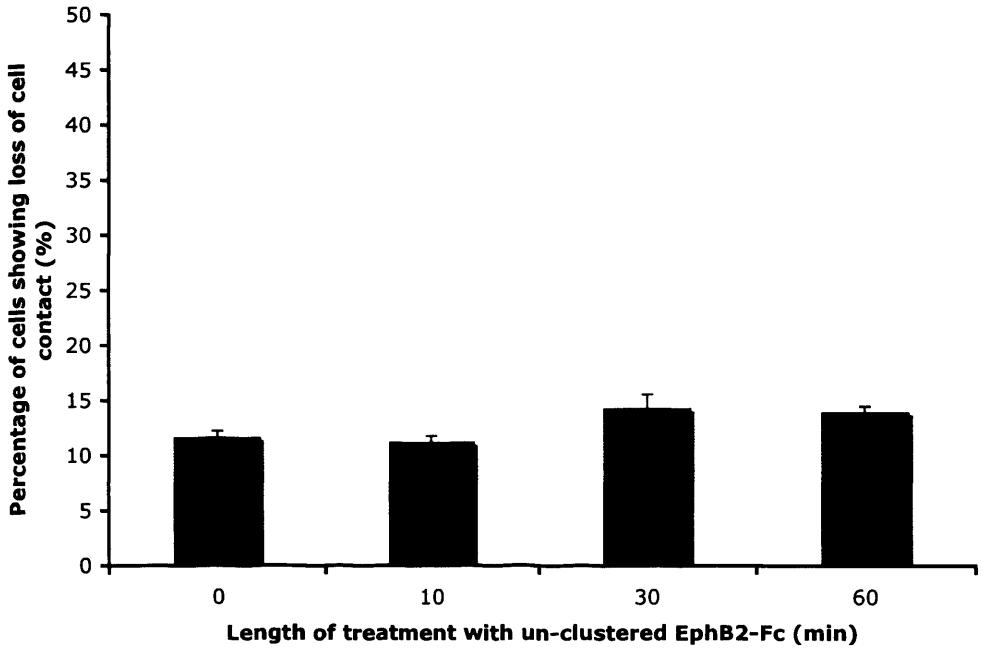


stimulation by EphB2-Fc, nor is the extent the same. This is most likely due to variation in the expression levels of ephrin-B2 achieved through microinjection. A certain threshold of expression may need to be reached in order for signalling to occur.

For the experiments described above, EphB2-Fc was pre-clustered prior to stimulation. However, Eph receptor activation by ephrin ligands is known to trigger different signalling pathways, depending on the level of clustering of the ephrin ligand. For example, human renal microvascular endothelial cells endogenously expressing EphB1, exhibit a decrease in cell attachment to fibronectin and receptor phosphorylation, when stimulated with ephrin-B1-Fc dimers. However, activation of EphB1 by stimulation with ephrin-B1-Fc pre-clustered into higher order aggregates causes cell attachment and capillary tube formation (Stein et al., 1998b). In order to see if this also applies to ephrin-B2 signalling, cells were stimulated with unclustered EphB2-Fc (1μg/ml) dimers. Interestingly, under these conditions there is no increase in loss of cell-cell contact compared with unstimulated cells (Fig. 3.5). It is therefore necessary to cluster the ligand into higher order aggregates using anti-human IgG in order to trigger loss of cell-cell contact.

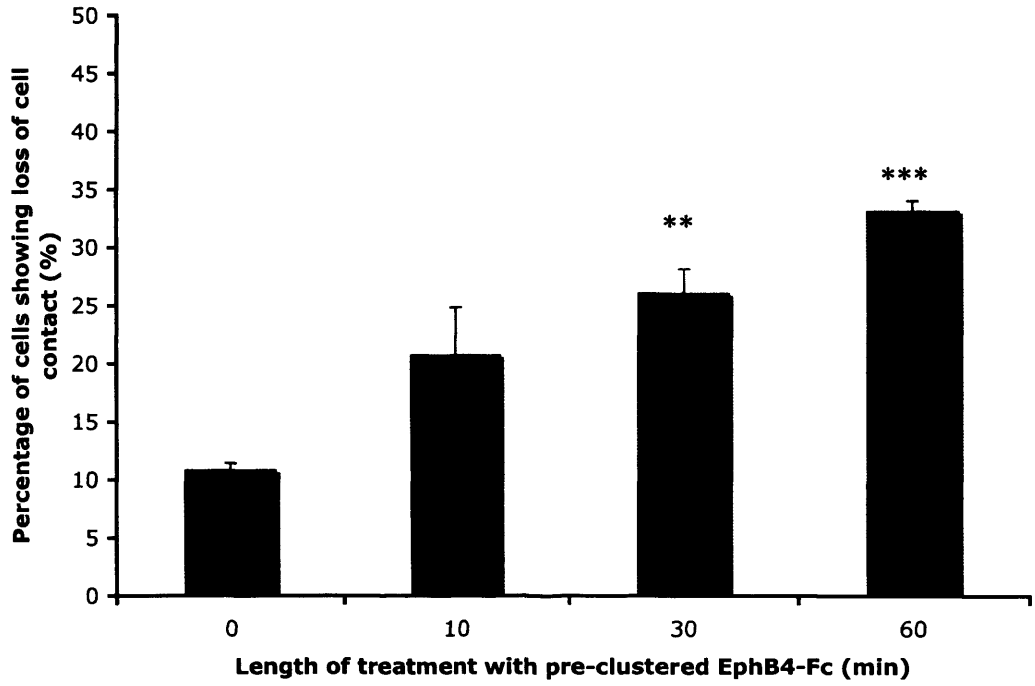
The loss of cell-cell contact response I observe is not specific to EphB2 stimulation. Ephrin-B2 expressing Swiss 3T3 cells stimulated with pre-clustered EphB4-Fc (1μg/ml), respond in a similar manner. The result is less dramatic than that seen upon EphB2-Fc stimulation, but there is an increased loss of cell-cell contact across the time course (Fig. 3.6). A significant proportion (>25%) of ephrin-B2 expressing cells have lost cell-cell contact after 30min stimulation (P <0.01; Fig. 3.6) rising to >30% after 60min stimulation (P <0.001; Fig.3.6). EphB4 is a known binding partner of ephrin-B2, and ephrin-B2 is in fact the only known ligand for EphB4 (Flanagan and Vanderhaeghen, 1998). Therefore, it is not surprising that EphB4 stimulation of ephrin-B2 induces a morphological response.

Figure 3.5 Stimulation of Swiss 3T3 cells expressing ephrin-B2 with un-clustered EphB2-Fc does not cause loss of cell-cell contact



Treatment with dimeric EphB2-Fc (1μg/ml) does not trigger ephrin-B2 expressing Swiss 3T3 cells to lose cell-cell contact compared with unstimulated cells. The experiment was repeated a minimum of 3 times with between 300-400 cells counted in total per time-point.

Figure 3.6 Stimulation of Swiss 3T3 fibroblasts expressing ephrin-B2 with pre-clustered EphB4-Fc results in loss of cell-cell contact



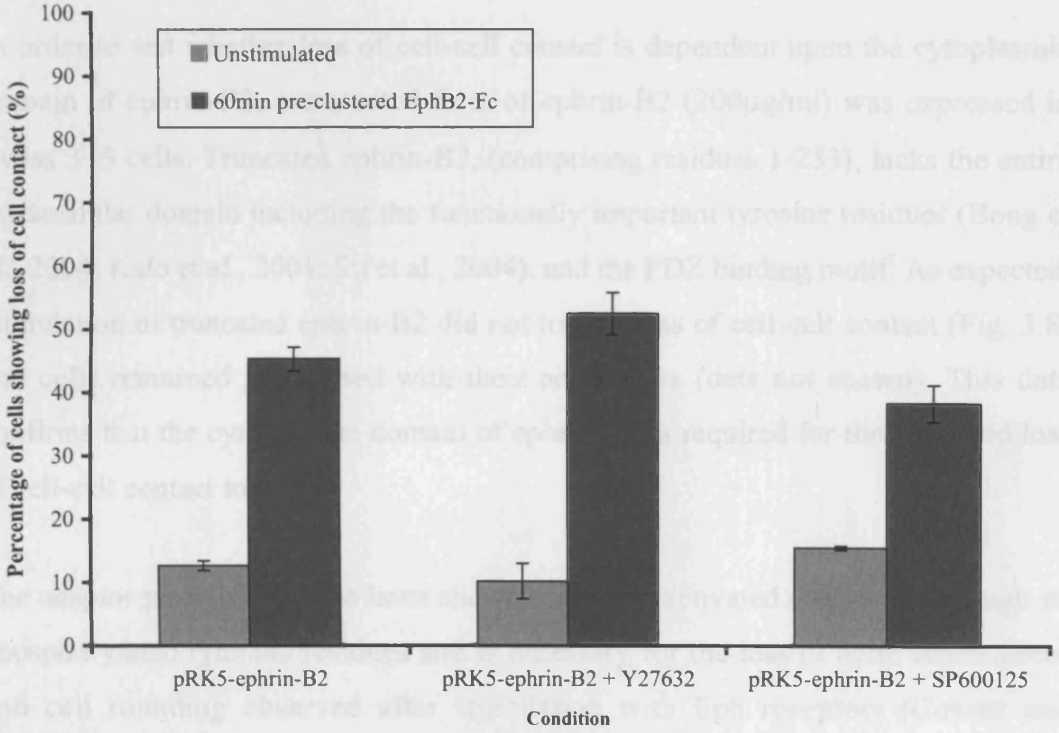
Ephrin-B2 expressing cells lose cell-cell contact upon treatment with pre-clustered EphB4-Fc (1μg/ml). Loss of cell-cell contact increases over time (ANOVA P = 0.0009) A significant proportion (26.9%) of ephrin-B2 expressing cells have lost cell-cell contact after 30min stimulation ($P < 0.01$). >30% cells have responded after 60min stimulation. The experiment was repeated a minimum of 3 times with between 300-400 cells counted in total per time point. (Asterisks denote statistical significance with respect to control).

3.2.4 Neither ROCK or JNK activity is required for loss of cell-cell contact induced by ephrin-B2 signalling

The Rho effector kinase, ROCK, and c-Jun amino terminal kinase (JNK) have both been implicated, independently, as signalling intermediates involved in a repulsive response to ephrin-B1 activation (Tanaka et al., 2003; Xu et al., 2003). In order to determine whether these signalling molecules are necessary for ephrin-B2 regulated loss of cell-cell contact, Swiss 3T3 fibroblasts were pre-treated with inhibitors to ROCK or JNK and the effect on loss of cell-cell contact investigated.

Treatment of ephrin-B2 expressing cells with the ROCK inhibitor Y27632 (20μM) for 30min caused confluent quiescent cells to lose actin stress fibres while remaining spread and in contact with their neighbours in the monolayer (data not shown). Y27632 did not inhibit EphB2-Fc triggered loss of cell-cell contact (Fig. 3.7) demonstrating that ROCK activity is not necessary for loss of cell-cell contact triggered as a result of ephrin-B2 clustering. Treatment of ephrin-B2 expressing cells with the JNK inhibitor SP600125 (30μM) for 1h had no visible affect on cell morphology, cells did not lose their actin stress fibres and remained well-spread and closely juxtaposed within the monolayer (data not shown). SP600125 did not inhibit loss of cell-cell contact triggered by EphB2-Fc (Fig. 3.7) therefore it is unlikely that JNK is involved in the loss of cell-cell contact observed after ephrin-B2 clustering in these cells.

Figure 3.7 Ephrin-B2 triggered loss of cell-cell contact does not require ROCK activity and is not blocked by the JNK inhibitor SP600125



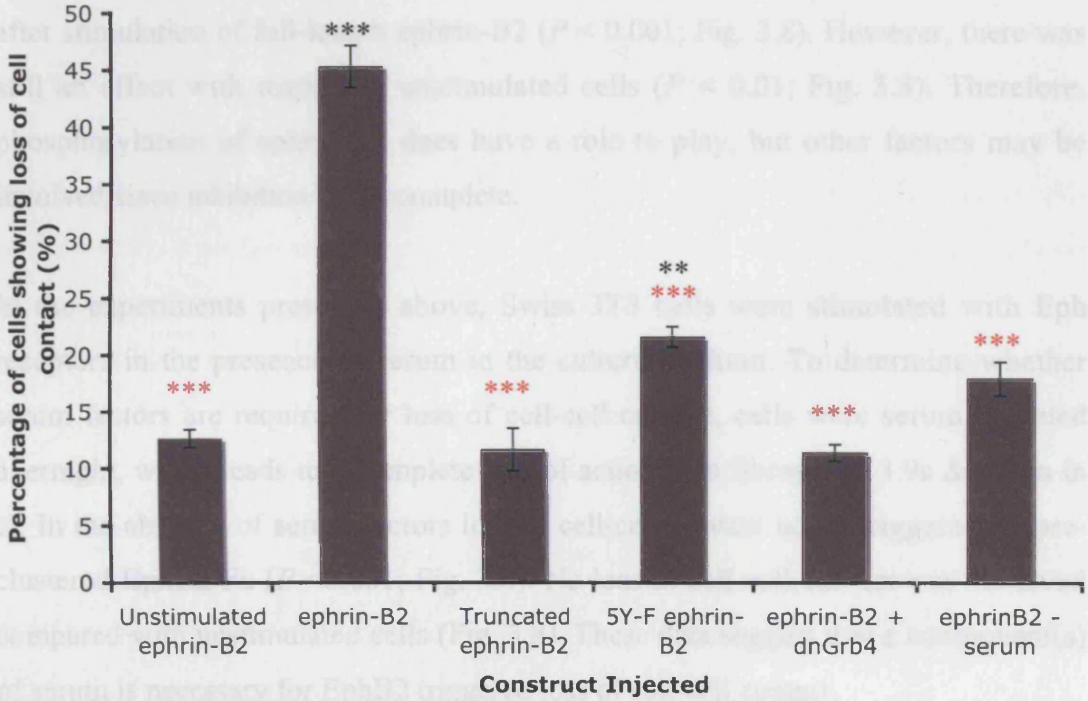
Swiss 3T3 cells exogenously expressing ephrin-B2 were untreated or pre-treated with the ROCK inhibitor Y27632 (20μM) or the JNK inhibitor SP600125 (30μM) prior to stimulation with pre-clustered EphB2-Fc (1μg/ml) for 1h. No significant difference in loss of cell-cell contact was observed compared to stimulation in the absence of these inhibitors. The experiment was repeated a minimum of 3 times, and between 300-400 cells were counted in total for each condition.

3.2.5 The cytoplasmic domain of ephrin-B2, tyrosine phosphorylation, Grb4 and serum factors are necessary for loss of cell-cell contact induced by ephrin-B2 clustering

In order to test whether loss of cell-cell contact is dependent upon the cytoplasmic domain of ephrin-B2, a truncated form of ephrin-B2 (200μg/ml) was expressed in Swiss 3T3 cells. Truncated ephrin-B2, (comprising residues 1-253), lacks the entire intracellular domain including the functionally important tyrosine residues (Bong et al., 2004; Kalo et al., 2001; Su et al., 2004), and the PDZ binding motif. As expected, stimulation of truncated ephrin-B2 did not trigger loss of cell-cell contact (Fig. 3.8) and cells remained juxtaposed with their neighbours (data not shown). This data confirms that the cytoplasmic domain of ephrin-B2 is required for the observed loss of cell-cell contact to occur.

The adaptor protein Grb4 has been shown to bind to activated ephrin-B1 through its phosphorylated tyrosine residues and is necessary for the loss of actin stress fibres and cell rounding observed after stimulation with Eph receptors (Cowan and Henkemeyer, 2001). In order to test whether Grb4 is an important component of ephrin-B2 signalling, dominant negative Grb4 (myc tagged; 200μg/ml) was co-expressed with ephrin-B2 (200μg/ml) in Swiss 3T3 fibroblasts and expression of dnGrb4 and ephrin-B2 detected using anti-myc and anti-ephrin-B2 antibodies respectively (data not shown). Dominant negative Grb4, comprising the isolated SH2 domain, can bind to tyrosine-phosphorylated ephrin-B2, which would prevent endogenous Grb4 from binding. Since this construct lacks SH3 domains it would inhibit downstream signalling by preventing the recruitment of other proteins implicated in reverse signalling (Cowan and Henkemeyer, 2001). Upon stimulation of cells co-expressing dnGrb4 and ephrin-B2 with pre-clustered EphB2-Fc, loss of cell-cell contact was inhibited to levels similar to those seen for unstimulated ephrin-B2 expressing cells ($P < 0.001$), demonstrating a requirement for Grb4 in signalling mediated by clustered ephrin-B2 (Fig. 3.8).

Figure 3.8 Loss of cell-cell contact exhibited by ephrin-B2 expressing cells under various conditions after 60min EphB2-Fc stimulation



Swiss 3T3 fibroblasts exogenously expressing ephrin-B2 lose cell-cell contact in response to stimulation with pre-clustered EphB2-Fc for 60min (P <0.001). Expression of truncated ephrin-B2 (lacking the intracellular region) does not trigger loss of cell-cell contact and co-expression of dominant negative Grb4 inhibits the response. Serum-starvation renders cells unable to lose contact in response to EphB2 stimulation. Expression of 5Y-F ephrin-B2 triggers only a weak loss of cell-cell contact response compared to full-length ephrin-B2 (P <0.001) but there is a significant increase with respect to control (P <0.01). Each experiment was repeated a minimum of 4 times, with between 500-600 cells counted in total for each condition. (* describes statistical difference with respect to unstimulated cells; * describes statistical difference with respect to full-length ephrin-B2 expressing cells).

In order to determine if phosphorylation of ephrin-B2 is important for loss of cell-cell contact a mutant ephrin-B2 construct, where all five tyrosine residues in the last 33 amino acids of the cytoplasmic tail have been mutated to phenylalanine, (5Y-F ephrinB2; 200μg/ml), was expressed in Swiss 3T3 fibroblasts. 5Y-F ephrin-B2 triggered a reduced loss of cell-cell contact response in comparison to that observed after stimulation of full-length ephrin-B2 ($P < 0.001$; Fig. 3.8). However, there was still an effect with respect to unstimulated cells ($P < 0.01$; Fig. 3.8). Therefore, phosphorylation of ephrin-B2 does have a role to play, but other factors may be involved since inhibition is not complete.

In the experiments presented above, Swiss 3T3 cells were stimulated with Eph receptors in the presence of serum in the culture medium. To determine whether serum factors are required for loss of cell-cell contact, cells were serum depleted overnight, which leads to a complete loss of actin stress fibres (Fig 3.9a & green in c). In the absence of serum factors loss of cell-cell contact is not triggered by pre-clustered EphB2-Fc ($P <0.001$; Fig. 3.8). No loss of cell-cell contact was observed compared with unstimulated cells (Fig. 3.8). These data suggest that a component(s) of serum is necessary for EphB2 triggered loss of cell-cell contact.

3.2.6 FGF is required for ephrin-B2 regulated loss of cell-cell contact in serum-starved cells

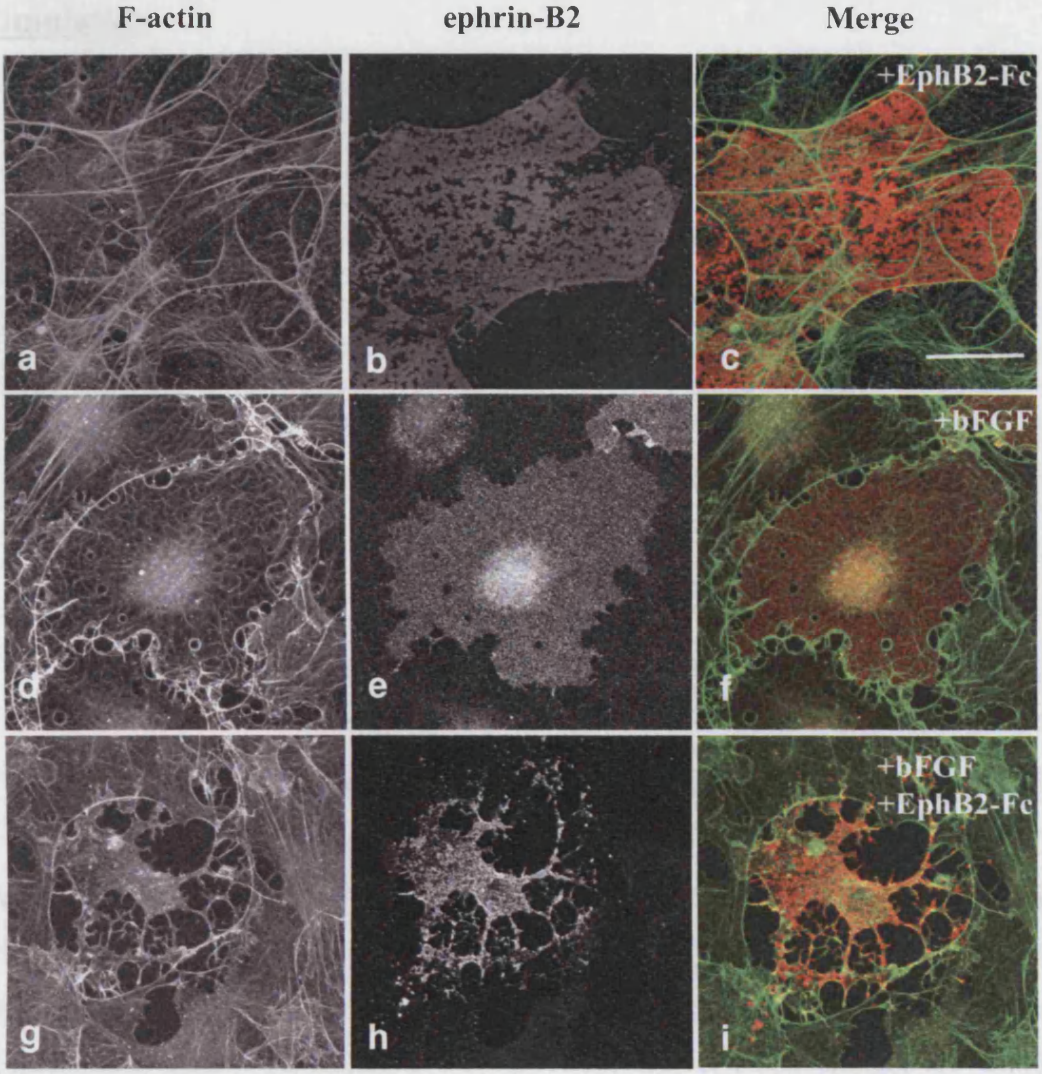
Since serum is important for loss of cell-cell contact triggered by ephrin-B2 clustering, I wanted to determine which factors in serum may be required for this ephrin-mediated response. An obvious candidate is the growth factor FGF, since it has previously been shown in *Xenopus* that FGFR can bind to and phosphorylate ephrin-B1 and regulate cell adhesion. An FGFR interaction with ephrin-B1 has also been shown to occur during cell migration into the eye field in *Xenopus* (Chong et al., 2000; Moore et al., 2004). To test the involvement of FGF, serum-starved confluent Swiss 3T3 cells were injected with an expression construct for ephrin-B2 (200μg/ml) and incubated at 37°C/10% CO_2 for 2h to allow for ephrin-B2 expression. After 1.5h cells were treated with 20ng/ml bFGF for 30min, followed by stimulation with pre-clustered EphB2-Fc (1μg/ml).

Under starved conditions pre-clustered EphB2-Fc stimulation of ephrin-B2 expressing Swiss 3T3 fibroblasts does not trigger loss of cell-cell contact (Fig. 3.8 & 3.9a-c). Treatment of ephrin-B2 expressing cells with bFGF in the absence of pre-clustered EphB2-Fc causes membrane ruffling (Ridley, 1995) but ephrin-B2 expressing cells do not lose cell-cell contact (Fig. 3.9d-f). However, upon treatment with bFGF followed by stimulation with pre-clustered receptor, ephrin-B2 expressing cells show a dramatic loss of cell-cell contact phenotype, with cell body rounding and the presence of long retraction fibres that remain in contact with non-ephrin-B2 expressing cells (Fig. 3.9g-i). There is a significant increase in loss of cell-cell contact across the time course (Fig. 3.10). Less than 10% of cells lose cell-cell contact without pre-clustered EphB2-Fc stimulation (time 0). After 10min treatment with pre-clustered EphB2-Fc 34% of expressing cells have lost cell-cell contact ($P < 0.05$; Fig. 3.10). This rises to more than 40% after 60min ($P < 0.001$; Fig. 3.10). Therefore, serum-starved cells require FGF treatment in order to round up in response to pre-clustered EphB2-Fc.

3.2.7 Loss of cell-cell contact triggered by ephrin-B2 clustering in the presence of FGF is dependent on Src tyrosine kinase activity, tyrosine phosphorylation of ephrin-B2 and Grb4 function

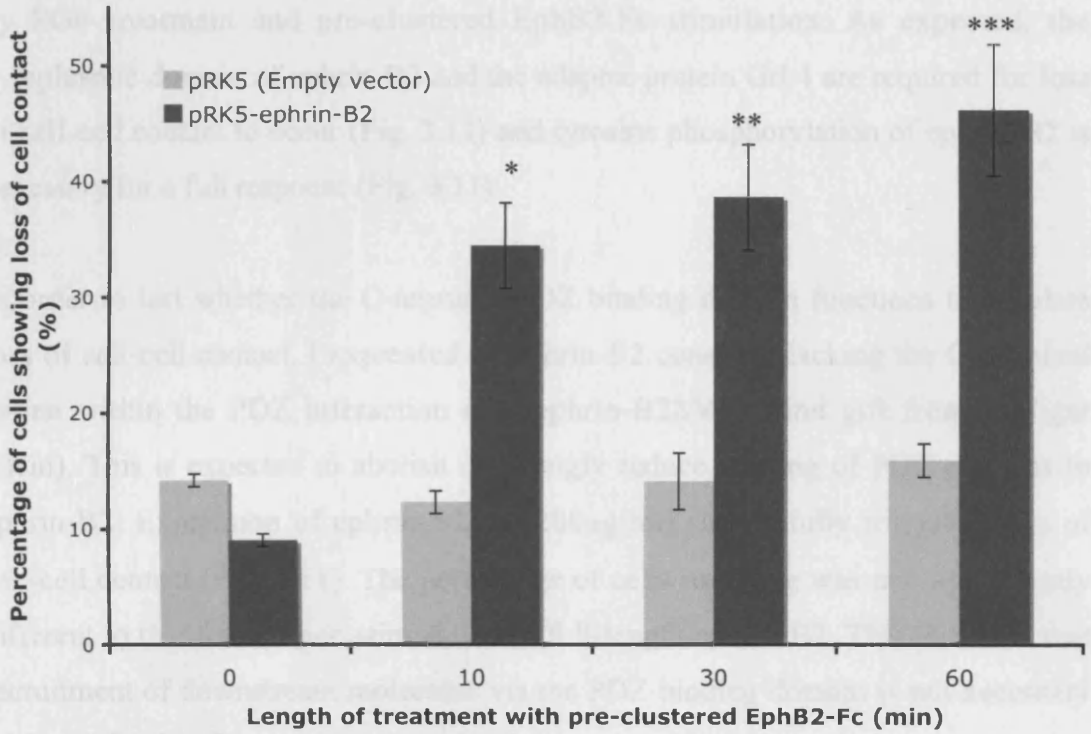
Src family kinases have been shown to mediate ephrin-B phosphorylation in primary endothelial cells and cortical neurons (Palmer et al., 2002). In order to determine if Src is involved in the loss of cell-cell contact I observe in response to ephrin-B2 clustering, ephrin-B2 expressing cells were serum-starved, treated with bFGF as described previously and pre-treated with the Src inhibitor PP2 (10μM) for 30min followed by stimulation with soluble pre-clustered EphB2-Fc. Inhibition of Src, using this pharmacological inhibitor, dramatically inhibits loss of cell-cell contact ($P < 0.001$; Fig. 3.11), indicating a requirement for Src family kinases in this EphB2 triggered loss of cell–cell contact.

Figure 3.9 FGF is required for EphB2-Fc induced ephrin-B2 signalling and subsequent loss of cell-cell contact



Cells were labelled for F-actin with FITC-phalloidin (green) and ephrin-B2 using an anti-ephrin-B2 antibody (red). Stimulation of serum-starved Swiss 3T3 cells expressing ephrin-B2 with pre-clustered EphB2-Fc alone (1μg/ml) for 60min caused clustering of ephrin-B2 into patches (b & red in c) but no loss of cell-cell contact (a & green in c). Treatment with bFGF (20ng/ml) alone for 30min caused reorganisation of actin into ruffles but no loss of cell-cell contact (d-f). Treatment with bFGF (20ng/ml) for 30min followed by pre-clustered EphB2-Fc stimulation for 60min caused clustering of ephrin-B2 into patches (h & red in i) and dramatic loss of cell-cell contact and cell rounding (g & green in i). The experiment was carried out four times with a minimum of 400 cells counted in total per time point. Scale bar 10μm.

Figure 3.10 Serum-starved ephrin-B2 expressing cells, in the presence of FGF, lose cell-cell contact in response to EphB2 stimulation



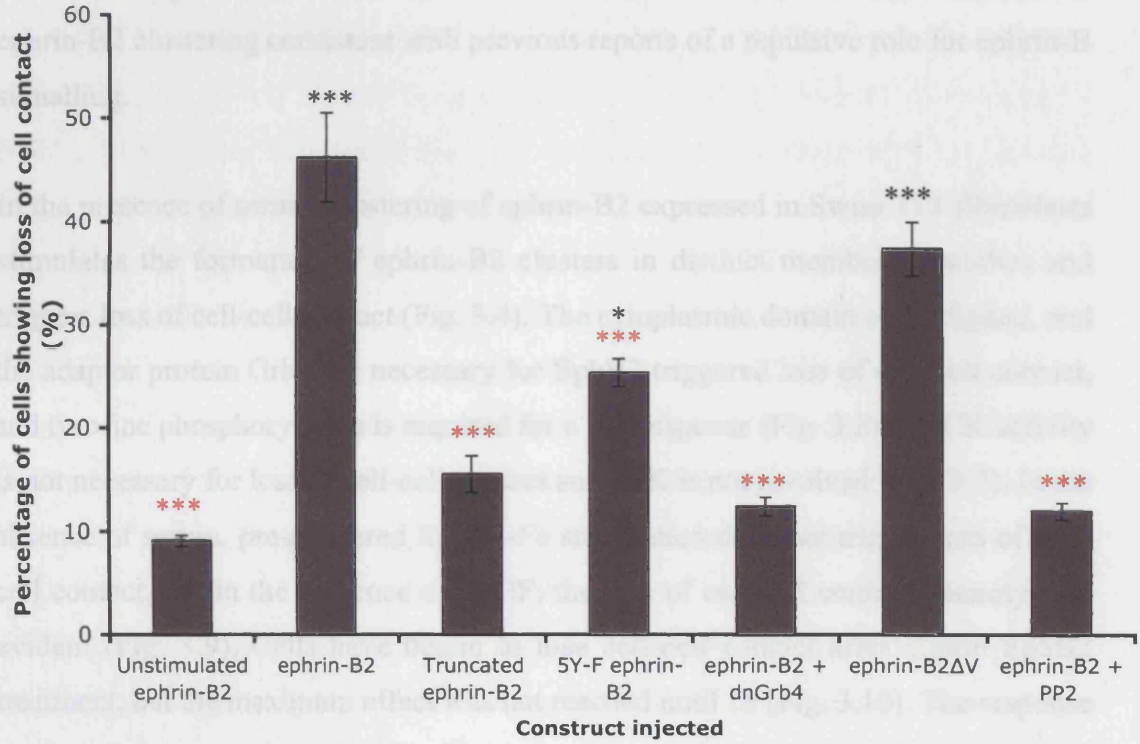
Swiss 3T3 fibroblasts exogenously expressing ephrin-B2 and subsequently treated with 20ng/ml bFGF lose cell-cell contact in response to stimulation with pre-clustered EphB2-Fc (ANOVA $P = 0.0003$). After 10min stimulation a significant proportion of ephrin-B2 expressing cells (34%) lose cell-cell contact ($P < 0.05$), rising to 46% after 1h ($P < 0.001$). Injection of pRK5 alone does not result in loss of cell-cell contact. The experiment was carried out a minimum of 3 times and between 300-500 cells were counted in total for each time point. (Asterisks denote statistical significance with respect to unstimulated cells).

As detailed previously (3.2.5) the cytoplasmic domain of ephrin-B2 is required for loss of cell-cell contact induced by ephrin-B2 clustering in the presence of serum. In order to test whether this is also true for serum-starved cells treated with FGF, mutant ephrin-B2 constructs were expressed as already described (3.2.5), followed by FGF treatment and pre-clustered EphB2-Fc stimulation. As expected, the cytoplasmic domain of ephrin-B2 and the adaptor protein Grb4 are required for loss of cell-cell contact to occur (Fig. 3.11) and tyrosine phosphorylation of ephrin-B2 is necessary for a full response (Fig. 3.11).

In order to test whether the C-terminal PDZ binding domain functions to regulate loss of cell-cell contact, I expressed an ephrin-B2 construct lacking the C-terminal valine within the PDZ interaction site (ephrin-B2ΔV; a kind gift from Rudiger Klein). This is expected to abolish or strongly reduce binding of PDZ proteins to ephrin-B2. Expression of ephrin-B2ΔV (200μg/ml) successfully triggered loss of cell-cell contact (Fig. 3.11). The percentage of cells rounding was not significantly different to that found upon stimulation of full-length ephrin-B2. This indicates that recruitment of downstream molecules via the PDZ binding domain is not necessary for loss of cell-cell contact triggered by Eph receptor binding.

Therefore, the cytoplasmic domain of ephrin-B2, tyrosine phosphorylation, Src tyrosine kinase activity and Grb4 are all required for loss of cell-cell contact in response to ephrin-B2 clustering, in the presence of FGF after serum-starvation (Fig. 3.11). These findings agree with those found upon stimulation of non-starved Swiss 3T3 cells with pre-clustered EphB2-Fc indicating that the same pathway is involved. PDZ protein interactions are not required for loss of cell-cell contact to occur.

Figure 3.11 Loss of cell-cell contact exhibited by serum-starved ephrin-B2 expressing cells, in the presence of FGF, after 60min EphB2-Fc stimulation under various conditions



Pre-treatment of ephrin-B2 expressing cells with 20ng/ml bFGF followed by stimulation with pre-clustered EphB2-Fc for 1h causes dramatic loss of cell-cell contact ($P < 0.001$). Expression of truncated ephrin-B2 does not trigger loss of cell-cell contact. Co-expression of dnGrb4 or pre-treatment with PP2, an inhibitor of Src family kinases, inhibits the loss of cell-cell contact. Expression of 5Y-F ephrin-B2 triggered a reduced loss of cell-cell contact response ($P < 0.001$), but there is an effect with respect to control ($P < 0.05$). Expression of ephrin-B2 containing a mutation in the PDZ binding domain does not significantly affect the loss of cell-cell contact response. Each experiment was repeated a minimum of 3 times and 300-500 cells counted in total for each condition. (* describes statistical significance with respect to unstimulated cells; * describes statistical significance with respect to full-length ephrin-B2 expressing cells).

3.3 Discussion

In this chapter, I have examined the response of confluent, quiescent Swiss 3T3 cells exogenously expressing ephrin-B2, to stimulation with soluble pre-clustered EphB2-Fc. Taken together the results demonstrate a loss of cell-cell contact response to ephrin-B2 clustering consistent with previous reports of a repulsive role for ephrin-B signalling.

In the presence of serum, clustering of ephrin-B2 expressed in Swiss 3T3 fibroblasts stimulates the formation of ephrin-B2 clusters in distinct membrane patches and triggers loss of cell-cell contact (Fig. 3.4). The cytoplasmic domain of the ligand, and the adaptor protein Grb4 are necessary for EphB2 triggered loss of cell-cell contact, and tyrosine phosphorylation is required for a full response (Fig. 3.8). ROCK activity is not necessary for loss of cell-cell contact and JNK is not involved (Fig. 3.7). In the absence of serum, pre-clustered EphB2-Fc stimulation does not trigger loss of cell-cell contact, but in the presence of bFGF, the loss of cell-cell contact phenotype is evident (Fig. 3.9). Cells have begun to lose cell-cell contact after 10min EphB2 treatment, but the maximum effect was not reached until 1h (Fig. 3.10). The response does not appear to be transient. The cytoplasmic domain of the ligand is again required for loss of cell-cell contact (Figs 3.8 & 3.11) as well as Src family kinases, Grb4 and tyrosine phosphorylation (Fig. 3.8 & 3.11). Recruitment of PDZ containing proteins (Fig. 3.11) is not necessary for loss of cell-cell contact triggered by ephrin-B2 clustering.

No morphological changes occur upon EphB2 stimulation of un-injected Swiss 3T3 fibroblasts, which I have shown to endogenously express ephrin-B3. One explanation for this is that the expression levels are not sufficient to induce a morphological response. Alternatively, ephrin-B3 activation may not trigger an actin cytoskeletal/morphological response, unlike ephrin-B1 (Cowan and Henkemeyer, 2001; Xu et al., 2003) and ephrin-B2 (described in this chapter).

3.3.1 Serum factors or FGF is necessary for loss of cell-cell contact triggered by ephrin-B2 clustering

Eph receptors and ephrins, as well as FGF receptor families and their activating ligands, have been shown to coordinate cell adhesion and migration during development (DeVore et al., 1995; Huynh-Do et al., 2002; Meyer et al., 2005; Osterhout et al., 1997). Ephrin-B ligands can become phosphorylated on tyrosine by both growth factor and EphB receptor treatment. Stimulation with the soluble ectodomain of Eph receptors has been shown to induce tyrosine phosphorylation of ephrin-Bs. For example, exogenously expressed ephrin-B1 in BHK cells becomes tyrosine phosphorylated upon treatment with EphB2 (Cowan and Henkemeyer, 2001), and endogenous ephrin-B in primary neurons or endothelial cells undergoes tyrosine phosphorylation upon treatment with EphB-Fc (Palmer et al., 2002). The FGF receptor has been shown to directly bind to and phosphorylate transmembrane ephrin-Bs and modulate their effect on cell adhesion. In *Xenopus* embryos, FGF receptor activation can inhibit the dissociative effects of x-ephrin-B1 (Chong et al., 2000). Also during retinal development in *Xenopus*, FGF modulation of ephrin-B reverse signalling is required to regulate the positioning of retinal progenitors within the eye (Moore et al., 2004). Platelet-derived growth factor (PDGF) is also capable of phosphorylating ephrin-Bs. Stimulation of the endogenous PDGF receptor in NIH3T3 fibroblasts ectopically expressing ephrin-B1 results in rapid ephrin-B1 phosphorylation. It is thought that ephrin-B1 could be an *in vivo* target for the PDGF receptor (Bruckner et al., 1997). Recent work has demonstrated a role for Eph/ephrin signalling in angiogenesis (reviewed in Heroult et al., 2005), and Tie-2, a member of a family of receptor tyrosine kinases known to function throughout angiogenesis, can phosphorylate ephrin-B1 *in vitro* (Adams et al., 1999). FGF signalling is also thought to play a role in the development of the vasculature (Wilkie et al., 1995). It is thought that cross-talk may exist between signalling pathways triggered by growth factors such as FGF and ephrin-B proteins. The data presented here support a role for cross-talk between FGF and ephrin-B signalling to regulate cell-cell contact.

There is currently no evidence for FGF involvement in ephrin-B signalling in mammals. However, the results in this chapter show that serum-starved Swiss 3T3

fibroblasts, exogenously expressing ephrin-B2, do not lose cell-cell contact in response to pre-clustered EphB2-Fc stimulation, unlike those in the presence of serum or FGF (Figs 3.8 & 3.9). Similarly, treatment with FGF alone does not cause ephrin-B2 expressing cells to lose cell-cell contact (Fig. 3.9). These results indicate FGF has a contributory effect to the loss of cell-cell contact observed, corroborating a role for FGFR and ephrin-B cross-talk to mediate cell-cell contact.

3.3.2 Src dependent phosphorylation of ephrin-B2 is required for EphB2 triggered loss of cell-cell contact in Swiss 3T3 fibroblasts

Ephrin-B ligands do not possess a catalytic domain, but phosphorylation of ephrin-Bs on tyrosine can be mediated by other kinases, such as the FGFR and Src (Chong et al., 2000; Palmer et al., 2002). For example, ephrin-B1 has been shown to be phosphorylated by Src *in vitro* upon Eph receptor engagement (Bruckner et al., 1997; Palmer et al., 2002). Ephrin-Bs are localised within lipid rafts (Bruckner et al., 1999) and Src family kinases and ephrin-B ligands co-localise in discrete signalling centres within lipid rafts after Eph receptor induced clustering (Palmer et al., 2002). Pre-treatment of Swiss 3T3 fibroblasts with PP2, a pharmacological inhibitor of Src family kinases, inhibits the loss of cell-cell contact I observe in response to ephrin-B2 clustering (Fig. 3.11). These data suggest the involvement of Src family kinases in the loss of cell-cell contact response. The pyrazolopyrimidine PP2 was developed as an inhibitor of the Src family and was first used to look at the effects of T cell receptor induced T cell activation, where the activity of the Src kinases Lck and FynT have been shown to be important (Hanke et al., 1996). Recently however, PP1 and PP2, although fairly selective, have been shown to inhibit other protein kinases as well, although less potently (Bain et al., 2003). This should be considered when interpreting the results as the involvement of Src family kinases has only been tested by pre-treatment with PP2. However, their involvement would corroborate the current literature.

Phosphorylation of ephrin-Bs on tyrosine is known to occur at specific tyrosine residues situated within the C-terminal 33 residues of the cytoplasmic tail, both *in vivo* and *in vitro* (Bruckner et al., 1997; Holland et al., 1996; Kalo et al., 2001). It

has been shown that this phosphorylation can occur both as a result of Eph receptor binding (Bruckner et al., 1997; Cowan and Henkemeyer, 2001) and through interactions with other receptor tyrosine kinases (Bruckner et al., 1997). Surprisingly, using a commercially available phospho-ephrin-B antibody or generic phosphotyrosine antibodies I was unable to detect phosphorylation of ephrin-B2 after Eph receptor treatment (results not shown). However, I did obtain evidence that suggests a requirement for tyrosine phosphorylation of ephrin-B2. 5Y-F ephrin-B2 is a construct that has all five tyrosine residues in the last 33 amino acids of the carboxy-terminal tail mutated to phenylalanine. Several studies have demonstrated the functional relevance of at least four out of five of the tyrosine residues found within the C-terminal 33 amino acids of B-ephrins. Tyr-312, Tyr-317 and Tyr-331 are phosphorylated upon Eph receptor engagement *in vivo* (Kalo et al., 2001) and Tyr-304 has been shown to be important for Grb4 binding via its SH2 domain (Su et al., 2004). Pre-clustered EphB2-Fc stimulation of expressed 5Y-F ephrin-B2 produced a much weaker loss of cell-cell contact response compared to stimulation of the full-length protein (Figs 3.8 & 3.11). This indicates that tyrosine phosphorylation is necessary for the full response to occur.

3.3.3 The adaptor protein Grb4 is required for ephrin-B2 regulated loss of cell-cell contact

It has been previously demonstrated in BHK cells that the SH2/SH3 domain adaptor protein Grb4 binds to the cytoplasmic domain of expressed ephrin-B1 in a phosphotyrosine dependent manner and this leads to cell rounding and actin cytoskeletal modifications (Cowan and Henkemeyer, 2001). However, cell rounding has also been demonstrated to occur independently of both tyrosine phosphorylation and Grb4 and in this instance was mediated by JNK (Xu et al., 2003). The results presented here would agree that Grb4 is necessary for loss of cell-cell contact in response to ephrin-B2 clustering. Co-expression of dnGrb4 together with ephrin-B2 reduced loss of cell-cell contact to control levels (Figs 3.8 & 3.11). This dominant negative construct retains the ability to bind to ephrin-B2 as it consists of the isolated SH2 domain. However, the ability to recruit downstream signalling molecules is impaired due to a lack of the functional SH3 domains. Grb4 is known to bind to

many proteins that have been implicated in the regulation of the cytoskeleton (Cowan and Henkemeyer, 2001). I have shown that Grb4 is necessary to initiate the recruitment of signalling molecules necessary for modifying the actin cytoskeleton enabling loss of cell-cell contact to occur in response to ephrin-B2 clustering in this system.

Although SFKs have been shown to phosphorylate ephrin-Bs, it is not known whether tyrosine phosphorylation by SFKs can modulate the binding of SH2 containing adaptor proteins such as Grb4. Interestingly, a recent study had shown that Grb4 binding in *Xenopus* embryos is dependent on activated FGF receptor (FGFR) (Bong et al., 2004). It has been shown that activated FGFR induces ephrin-B1 tyrosine phosphorylation (Bong et al., 2004; Chong et al., 2000), and promotes the formation of a complex between ephrin-B1 and Grb4 (Bong et al., 2004). Binding of Grb4 to ephrin-B1 is phosphorylation dependent (Bong et al., 2004; Cowan and Henkemeyer, 2001), and is unable to occur in the presence of a kinase inactive FGFR (Bong et al., 2004). This would suggest that the requirement for FGF in this system is to facilitate Grb4 binding.

3.3.4 PDZ domain interactions are not required for loss of cell-cell contact triggered by ephrin-B2 activation

Numerous studies have demonstrated a functional role for the cytoplasmic domain in the propagation of the reverse signal (Cowan and Henkemeyer, 2001; Lin et al., 1999; Song et al., 2002; Xu et al., 2003). As described previously the last 33 amino acids of the C-terminal region of ephrin-Bs contain functionally important tyrosine residues, and at the extreme carboxy terminus a PDZ binding domain. Numerous PDZ domain interactions have been demonstrated to occur upon ephrin-B activation, although their function remains unclear (Bruckner et al., 1999; Lu et al., 2001; Palmer et al., 2002). As expected, loss of cell-cell contact in response to ephrin-B2 clustering is attributable to the cytoplasmic domain of the ligand (Figs 3.8 & 3.11). However, there was no significant reduction in loss of cell-cell contact observed when an ephrin-B2 construct lacking the C-terminal valine within the PDZ binding

motif was expressed (ephrin-B2ΔV; Fig. 3.11) indicating that PDZ binding domain interactions are not required for EphB2 triggered loss of cell-cell contact.

3.3.5 Rho Kinase and c-Jun amino terminal kinase are not required for loss of cell-cell contact.

The small GTPase Rho and the MAP kinase JNK have both been implicated in separate publications as regulators of cell rounding responses triggered by ephrin-B reverse signalling (Tanaka et al., 2003; Xu et al., 2003). JNK has been shown to be phosphorylated when both the forward and reverse signalling pathways are activated (Huynh-Do et al., 2002; Stein et al., 1998a) and overexpression of ephrin-B1 in human embryonic kidney (HEK) 293T cells dramatically increases the activity of JNK leading to a cell rounding response that is dependent on JNK activity (Xu et al., 2003). In *Xenopus,* the cytoplasmic protein Dishevelled (Xdsh) has been shown to mediate reverse signalling by EphB2 stimulated ephrin-B1 leading to the activation of RhoA and ROCK (Tanaka et al., 2003). However, the loss of cell-cell contact I observe here is not affected by treatment with inhibitors to either JNK or ROCK (Fig. 3.7) indicating that this loss of cell-cell contact is not a Rho kinase/acto-myosin driven contraction event and is not dependent on JNK activity.

3.3.6 Loss of cell-cell contact triggered by ephrin-B2 signalling requires EphB2 receptor clustering

The binding of ephrins to Eph receptors occurs at sites of cell-cell contact, and is known to cause clustering of both the ligands and receptors, which triggers signalling in both the receptor and ligand expressing cells (Kullander and Klein, 2002). In support of a requirement for clustering, ephrins were found to be inactive in soluble form, despite being able to activate receptors when presented on the surface of cells (Davis et al., 1994), suggesting that membrane attachment may facilitate ligand dimerisation or clustering, which may then promote receptor clustering. In support of this, artificial clustering enabled previously inactive soluble ligands to effectively tyrosine phosphorylate their cognate Eph receptors (Davis et al., 1994).

Stimulation of ephrin-B2 expressing cells with unclustered EphB2-Fc dimers did not cause loss of cell-cell contact to occur (Fig. 3.5). The extent of clustering required for ephrin ligands to activate their binding partners varies between receptor-ligand pairs, although it is thought that EphB receptors may have a higher requirement for aggregation beyond the dimerisation stage to become activated than EphAs (Gale and Yancopoulos, 1997). Since different levels of ligand clustering is known to affect receptor signalling, resulting in different cellular responses depending whether the ligand is presented as a dimer or higher order aggregate (Stein et al., 1998b), it is not surprising that the level of Eph receptor clustering affects signalling from the ephrins. Activation of ephrins and Eph receptors leads to the multimerisation of both molecules into plasma membrane clusters (Davis et al., 1994). The formation of clusters is thought to be necessary to initiate conformational changes that allow the subsequent phosphorylation and binding of signalling partners (Himanen et al., 2001; Song, 2003; Toth et al., 2001). Interactions between ephrins and Eph receptors occur at sites of cell-cell contact *in vivo* where assembly in lipid rafts is believed to occur as a consequence of cell-cell contact. Artificially clustering the receptor is simply further mimicking the *in vivo* process.

3.3.7 Conclusion

In response to EphB2-Fc stimulation of expressed ephrin-B2 in Swiss 3T3 fibroblasts, I observe a loss of cell-cell contact response requiring the presence of serum factors or FGF, the adaptor protein Grb4, Src family kinases and tyrosine phosphorylation of ephrin-B2. PDZ domain protein interactions are not required, the response is not dependent on ROCK activity, and does not involve JNK.

Cell rounding has been demonstrated to occur as a result of ephrin-B1 activation by two independent groups (Cowan and Henkemeyer, 2001; Xu et al., 2003). In one instance cell rounding is dependent on tyrosine phosphorylation and Grb4 binding (Cowan and Henkemeyer, 2001), in the other cell rounding can occur independently of Grb4, tyrosine phosphorylation and PDZ protein interactions, yet requires JNK activity (Xu et al., 2003). Taken together, the results presented here corroborate those of Cowan and Henkemeyer (Cowan and Henkemeyer, 2001). The loss of cell-cell

contact response I observe to ephrin-B2 clustering is dependent on Grb4 and tyrosine phosphorylation and does not require JNK activity.

However, there are key differences. I have demonstrated a novel loss of cell-cell contact phenotype as a result of ephrin-B2 clustering. In addition, I have provided evidence of a requirement for serum factors in order for loss of cell-cell contact in response to EphB2-Fc stimulation, where as cell rounding occurred after ephrin-B1 activation in serum-starved BHK cells. However, serum-starving BHK cells does not cause a complete loss of actin stress fibres, which is the case in Swiss 3T3 cells, and there is a low level of tyrosine phosphorylation seen in the unstimulated cells, indicating these cells may not be truly growth factor free. There is also a marked difference in the time course of events. The cell rounding response to expressed ephrin-B1 activation in BHK cells occurs over a time period of up to 6h. However, the loss of cell-cell contact response I observe in Swiss 3T3 cells upon clustering of expressed ephrin-B2 occurs after 10min reaching a maximum within 1h. This could be due to the expression levels of protein achieved, since ephrin-B1 was expressed in BHK cells by transfection, and microinjection was used to express ephrin-B2 in Swiss 3T3 fibroblasts. Alternatively the response may be cell type dependent. In addition, I have shown that PDZ domain protein interactions, which were not investigated by Cowan and Henkemeyer (Cowan and Henkemeyer, 2001), are not required for loss of cell-cell contact in response to ephrin-B2 clustering. This finding corroborates Xu and colleagues, who showed that the cell rounding response to ephrin-B1 activation in HEK 293 cells was independent of PDZ protein interactions (Xu et al., 2003). It is apparent that cell rounding as a consequence of ephrin-B clustering, can be mediated by two independent pathways.

Chapter 4

Ephrin-B2 signalling in endothelial cells results in membrane retraction and blebbing

4.1 Introduction

In chapter 3, I showed that loss of cell-cell contact occurred in response to stimulation of Swiss 3T3 fibroblasts, expressing exogenous ephrin-B2, with soluble pre-clustered EphB receptors. Having characterised the effects of ephrin-B2 signalling in this model system and the effects of receptor stimulation of various mutant ephrin-B2 constructs, I have now investigated the response of human endothelial cells, that endogenously express ephrin-B ligands, to EphB treatment.

Human Umbilical Arterial Endothelial Cells (HUAECs), endogenously expressing ephrin-Bs, have been used as a primary cell line to study reverse signalling in a physiologically relevant cell type. The aim was to provide an insight into how ephrin-B reverse signalling contributes to the regulation of cell movements necessary for the correct migration of endothelial cells throughout development.

Blood vessel development occurs via a number of different processes. Initially, through a process called vasculogenesis, endothelial cells differentiate and proliferate, merging to form a tubular network. This is followed by angiogenesis, which is the process of remodelling the primary vascular plexus to form the mature vascular network. This involves sprouting of new blood vessels as well as splitting and pruning of existing vessels to generate a larger branched network (Risau, 1997).

Angiogenesis requires the accurate migration of endothelial cells to specific target sites. There are many critical growth factors required for the regulation of blood vessel formation and several studies have implicated Eph receptors and ephrins (reviewed in Adams, 2002; Cheng et al., 2002a). *In vivo,* venous endothelial cells, expressing EphB4, and arterial endothelial cells expressing ephrin-B2, make contact in fine capillary beds during development. It is thought that the EphB4-ephrin-B2 interaction inhibits intermingling of the venous and arterial endothelial cells, and in this way maintains arterial-venous distinction, presumably via repulsive signals establishing and maintaining boundaries between the vessels (Wang et al., 1998). Deletion of ephrin-B2 or EphB4 results in identical defects in angiogenic remodelling and the phenotype is embryonic lethal (Adams et al., 1999; Gerety et al.,

1999; Wang et al., 1998). Ephrin-B reverse signalling, and not simply forward signalling from the receptors, was confirmed to be required when expression of a truncated form of ephrin-B2, lacking the cytoplasmic domain, failed to rescue the angiogenesis defects associated with loss of ephrin-B2 (Adams et al., 2001). However, this observation was challenged in a later transgenic mouse study where the cytoplasmic domain of ephrin-B2 was replaced by ß-galactosidase and no angiogenesis defects were observed (Cowan et al., 2004), demonstrating that the C-terminal domain of ephrin-B2 was not required during early vascular development. The differences between these two studies may simply be due to the type of mutations used leading to altered protein localisation, or possibly affecting their ability to cluster and function. Interestingly, mice with the ß-galactosidase insertion in place of the cytoplasmic domain die from cardiac defects postnatally, indicating a potential role for ephrin-B2 in heart development (Cowan et al., 2004).

Ephrin-B2 continues to selectively mark arteries during later embryonic development as well as in the adult, although expression extends to the surrounding arterial smooth muscle (Gale et al., 2001; Shin et al., 2001a). Therefore, ephrin-B2 is apparently not only required during the earliest stages of arterial/venous determination, but may be important during the development of arteries, perhaps by regulating interactions between endothelial smooth muscle cells involved in the formation of arterial walls (Yancopoulos et al., 2000). During angiogenesis in the adult, such as tumours or the female reproductive tract, the endothelium of new vessels re-expresses ephrin-B2 suggesting that ephrin-B2 may be important in these angiogenic settings (Yancopoulos et al., 2000).

In contrast to the repulsive signalling described above, ephrin-B2 can also induce capillary sprouting (Adams et al., 1999; Fuller et al., 2003; Palmer et al., 2002). For example, *in vivo,* ephrin-B2 deficient embryos show defective vascularisation of the nervous system, which would normally occur via angiogenic sprouting from the perineural vascular plexus (Adams et al., 1999). *In vitro,* ephrin-B2 has been shown to play a role in endothelial cell sprouting (Fuller et al., 2003).

Ephrin-B2 signalling has recently been implicated, not only in the development of blood vessels but also the lymphatic system (Makinen et al., 2005). The major roles

of the lymphatic system are to maintain tissue fluid balance, provide a route for fat absorption in the gut and immune surveillance. Lymphatic vessels appear to originate from a subset of venous endothelial cells and like the blood vasculature the lymphatic system undergoes remodelling. However, the development of the lymphatic vasculature is less understood. Makinen and colleagues, using knock-in mice, demonstrated a requirement *in vivo* for the PDZ binding motif at the C-terminal end of ephrin-B2 in order for correct lymphangiogenic remodelling (Makinen et al., 2005). Two knock-in mice were generated; Ephrin-B2$^{\Delta V}$ mice expressed ephrin-B2 lacking the C-terminal valine in the PDZ binding site and ephrin-B2^{5F} expressed ephrin-B2 with all 5 conserved tyrosine residues mutated to phenylalanine. Homozygous mutant mice survived the requirement for ephrin-B2 in the vascular system, however, ephrin-B2$^{\Delta V}$ exhibited major lymphatic defects (Makinen et al., 2005). Interaction with PDZ proteins, but not tyrosine phosphorylation, was found to be required for lymphatic remodelling.

Despite the lack of a catalytic domain, ephrin-Bs are capable of transducing a signal (reverse signalling). There is *in vitro* evidence that this ephrin-B signalling requires the cytoplasmic domain of the ligand (Adams et al., 2001; Xu et al., 2003). Ephrin-B ligands become phosphorylated on tyrosine upon Eph receptor engagement (Bong et al., 2004; Bruckner et al., 1997; Cowan and Henkemeyer, 2001; Holland et al., 1996; Kalo and Pasquale, 1999a; Su et al., 2004), and binding of the adaptor protein Grb4 has been shown to occur in a phosphotyrosine-dependent manner, linking ephrin-Bs to a range of molecules involved in actin cytoskeletal regulation, via its SH3 domains (Bong et al., 2004; Cowan and Henkemeyer, 2001; Su et al., 2004). Src family kinases have been shown to be responsible for tyrosine phosphorylation of the ligand upon Eph receptor binding (Bruckner et al., 1997; Holland et al., 1996; Palmer et al., 2002). In addition, tyrosine phosphorylation of ephrin-B1 can be induced independently of EphB receptor binding by PDGF and FGF (Bong et al., 2004; Bruckner et al., 1997; Chong et al., 2000; Palmer et al., 2002). The PDZ binding domain present in the cytoplasmic tail of all known ephrin-Bs has also been shown to mediate PDZ protein interactions *in vitro* (Lin et al., 1999; Lu et al., 2001; Torres et al., 1998).

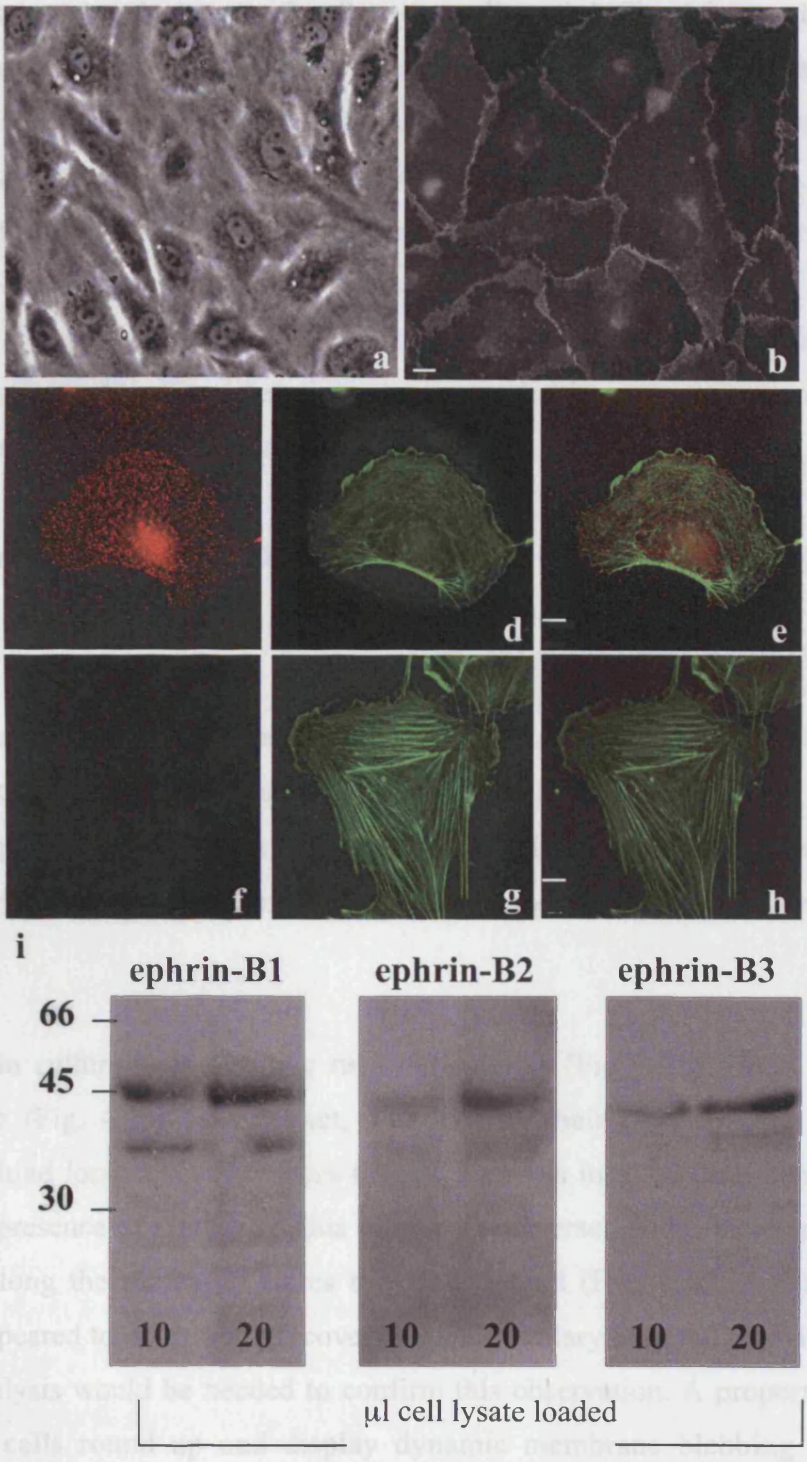
In this chapter, I show that EphB4-Fc stimulation of HUAECs triggers cell contraction, cell rounding and, in a proportion of the retracting cells, dynamic membrane blebbing. This response is transient, with cells recovering to a spread state within 30min, despite the continued presence of EphB4-Fc in the culture medium. Retraction requires the cytoplasmic domain of the ligand, but does not require the SH2/SH3 domain adaptor protein Grb4.

4.2 Results

4.2.1 Characterisation of HUAECs

Cultured arterial endothelial cells (HUAECs) have a flat morphology and form a monolayer, when grown to confluency on glass coverslips or plastic culture dishes (Fig. 4.1a), with adherens junctions between cells (Fig. 4.1b). Signalling from ephrin-B2 and its cognate receptor EphB4 has been implicated in regulating the formation and patterning of blood vessels throughout embryonic development (Adams et al., 1999; Gale et al., 2001; Gerety et al., 1999; Shin et al., 2001a; Wang et al., 1998). Using immunocytochemistry, I have confirmed that HUAECs endogenously express ephrin-B2 (Fig. 4.1c & red in e). HUAECs in culture were treated with pre-clustered EphB4-Fc that binds endogenous ephrin-B2, since this is the only known ligand for EphB4 (Kim et al., 2002). The cells were fixed after 20min pre-clustered EphB4-Fc stimulation and bound receptor was detected with fluorescent secondary antibodies. Punctate ephrin-B2 staining was observed, extending to the edge of the lamellae (Fig. 4.1c & red in e). Panels f-h are controls for non-specific antibody binding. Cells treated with the isolated Fc portion of human IgG pre-clustered with anti-human IgG, show no visible staining (Fig. 4.1f). Ephrin-B2 expression was confirmed by western blotting of cell lysates, using commercially available antibodies raised against the individual ephrin-Bs. HUAECs were found to endogenously express all 3 members of the ephrin-B family: ephrin-B1, ephrin-B2 and ephrin-B3 (Fig. 4.1i). But as noted above, stimulation of HUAECs with EphB4-Fc should activate only ephrin-B2 (Kim et al., 2002).

Figure 4.1 HUAECs express ephrin-B1, ephrin-B2 and ephrin-B3



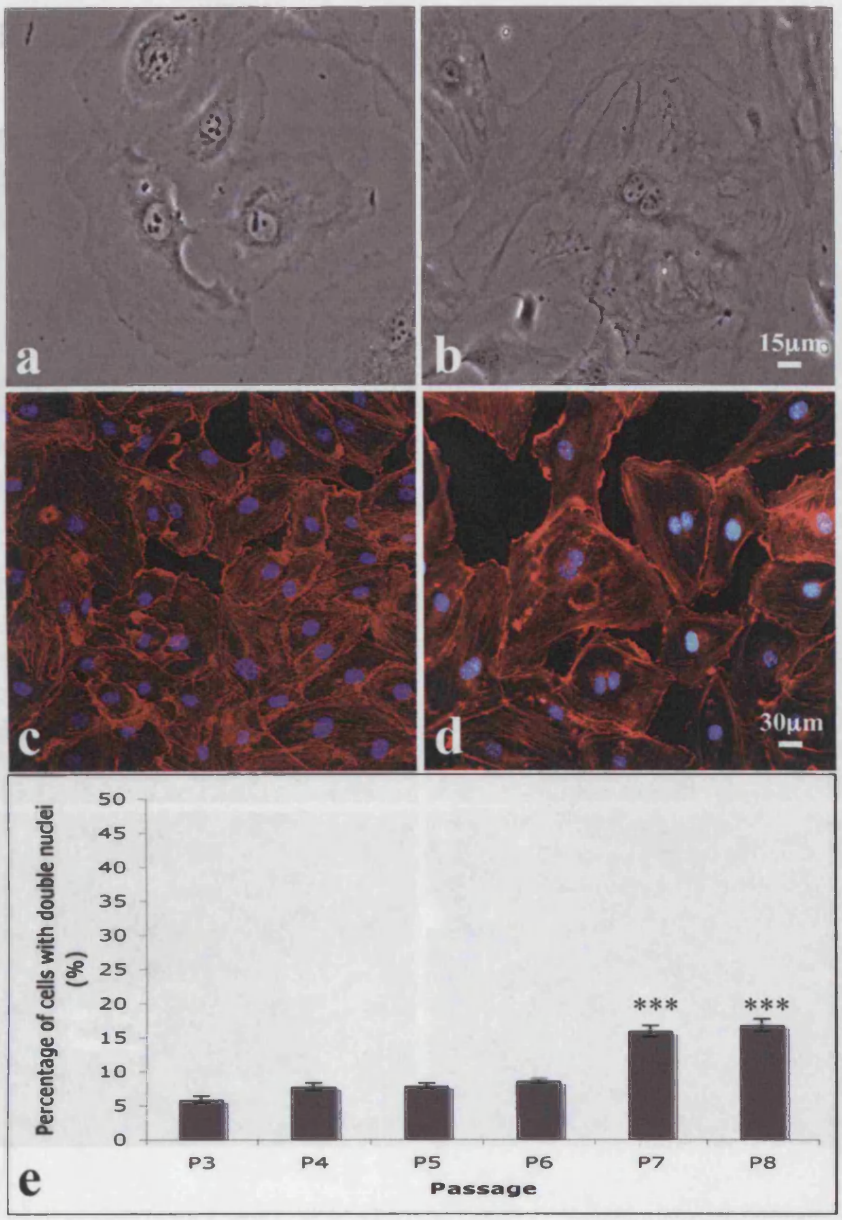
HUAECs were obtained as passage 1 stocks from TCS cellworks and cells between passages 3 and 6 were used for experiments, since those cultured beyond P6 showed signs of senescence. At passage 3 cells were well-spread (Fig. 4.2a), and displayed numerous actin stress fibres and membrane ruffles, demonstrated by staining for filamentous actin using TRITC-conjugated phalloidin (Fig. 4.2c). By passage 7/8 cells are larger and flatter (Fig. 4.2b&d) and their growth rate has slowed as demonstrated by the increased time between passaging. Higher passage cells displayed an increased incidence of multiple nuclei per cell (Fig. 4.2d&e). At passage 3, 6% of cells had double nuclei, whereas >15% of cells at passage 7 were bi-nucleate ($P < 0.001$; Fig. 4.2e). For consistent, reliable results cells were therefore used before the onset of senescence.

4.2.2 Stimulation of HUAECs with EphB4-Fc triggers a dramatic cell retraction response

In order to characterise the response of HUAECs to ephrin-B2 signalling, sub-confluent cells were stimulated with pre-clustered EphB4-Fc (5μg/ml; 2:1 molar ratio of EphB4-Fc:anti-human IgG) and the dynamic responses observed were recorded by time-lapse phase microscopy (Supplementary Material: Movies 4.1 & 4.2).

HUAECs in culture have dynamic ruffling lamellae (Fig. 4.3a). Upon EphB4-Fc stimulation (Fig. 4.3b) cells retract, withdrawing their margins (Fig. 4.3c) and leaving behind long retraction fibres (Fig. 4.3 arrows in d). Within 20min, in the continued presence of EphB4-Fc, this response is reversed and cells re-spread their lamellae along the retraction fibres they left behind (Fig. 4.3f). An increase in ruffling appeared to occur upon recovery (Supplementary Material: Movie 4.1), but further analysis would be needed to confirm this observation. A proportion of the retracting cells round up and display dynamic membrane blebbing (Fig. 4.4; Supplementary Material: Movie 4.2) characteristic of apoptotic cells. These cells also rapidly recover to a fully spread state within 20-40min (Fig 4.4; Supplementary Material: Movie 4.2).

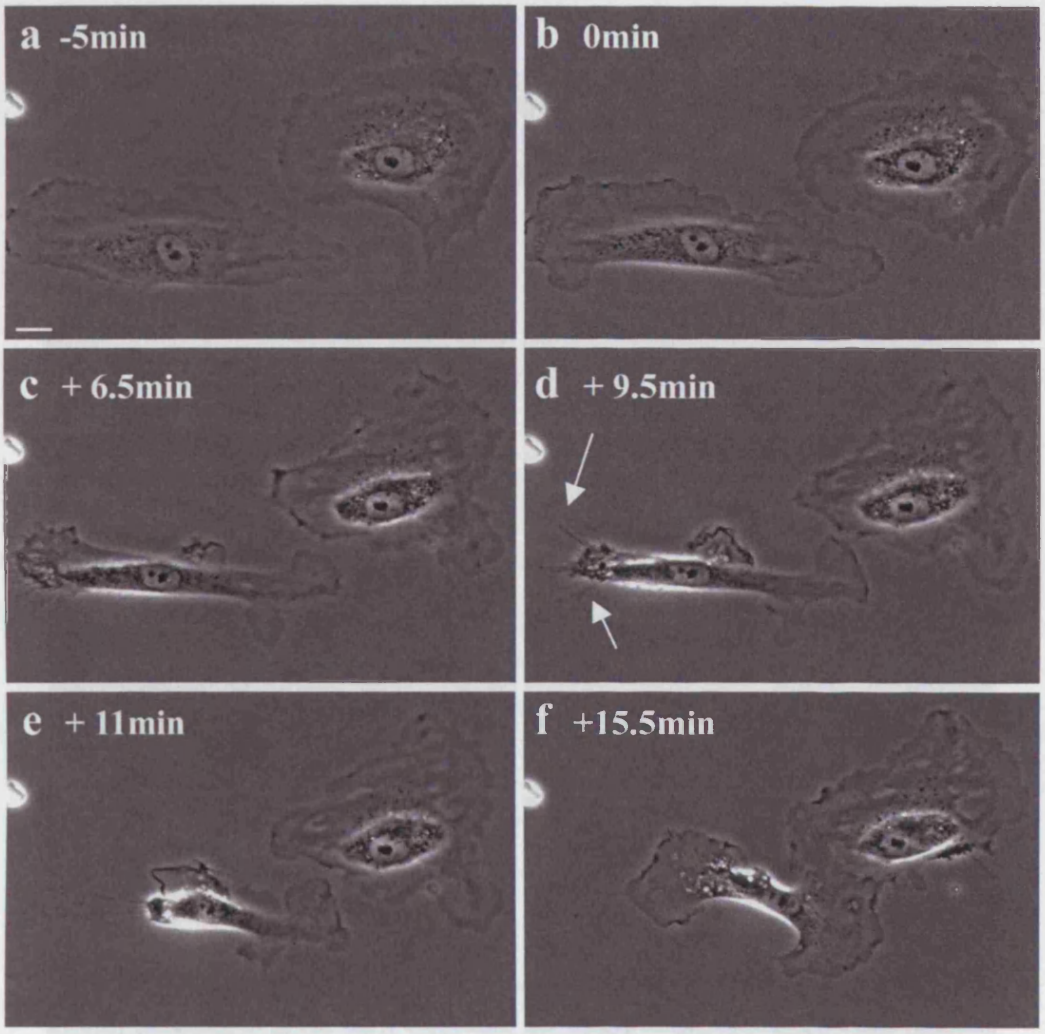
Figure 4.2 After passage 6 increasing numbers of HUAECs appear senescent



Cells were stained with TRITC-phalloidin to detect F-actin (red) and DAPI to detect cell nuclei (blue). Passage 3 HUAECs in culture have a well-spread, flat morphology (a) and display actin stress fibres (c). After passage 6 cells became larger, flatter (b) and appeared to display fewer actin stress fibres (d). >15% cells contain double nuclei after passage 6 compared with only 5% at passage 3 ($P < 0.001$; e). (Asterisks refer to the increase in double nuclei compared with P3 cells).

Figure 4.3 HUAECs stimulated with pre-clustered EphB4-Fc retract then rapidly re-spread

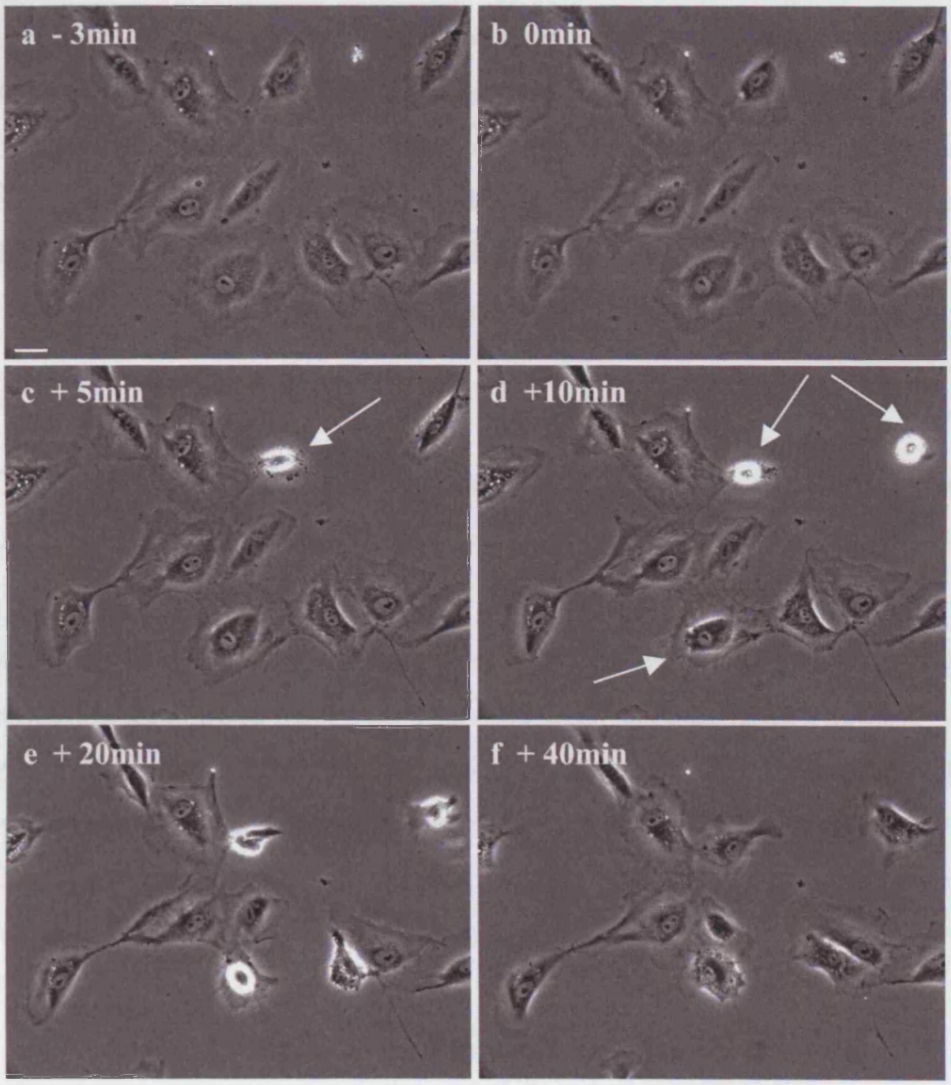
x40 magnification (retraction)



Phase time-lapse stills of HUAECs stimulated with pre-clustered EphB4-Fc (5μg/ml). Unstimulated HUAECs are well-spread and ruffling (a) Following stimulation cells retract (c-e), and leave behind long retraction fibres (c-e; arrows in d). Within 20min cell retraction is rapidly reversed and cells recover to display large membrane ruffles (f). Scale bar 15μm. (See Supplementary Material: Movie 4.1).

Figure 4.4 HUAECs stimulated with pre-clustered EphB4-Fc retract and a subset of cells undergo membrane blebbing, followed by rapid recovery

x20 Magnification (Blebbing)



Phase time-lapse stills of HUAECs stimulated with pre-clustered EphB4-Fc (5μg/ml). Unstimulated HUAECs are well-spread with ruffling lamella (a). Following EphB4-Fc stimulation cells retract and a proportion exhibit membrane blebbing (arrows in c&d). The effect is transient, after 20min EphB4-Fc stimulation cells recover and re-spread lamellae (e&f). Scale bar 30μm. (See Supplementary Material: Movie 4.2).

Using phase time-lapse microscopy of HUAECs in culture I have therefore revealed a transient cell retraction response to stimulation with pre-clustered EphB4-Fc. A series of distinct cellular events are observed. Initially cells round up, withdrawing their margins, leaving behind long retraction fibres, and a subset of cells undergo membrane blebbing. Retraction and blebbing are followed by rapid recovery to a fully spread state, despite the continued presence of EphB4-Fc.

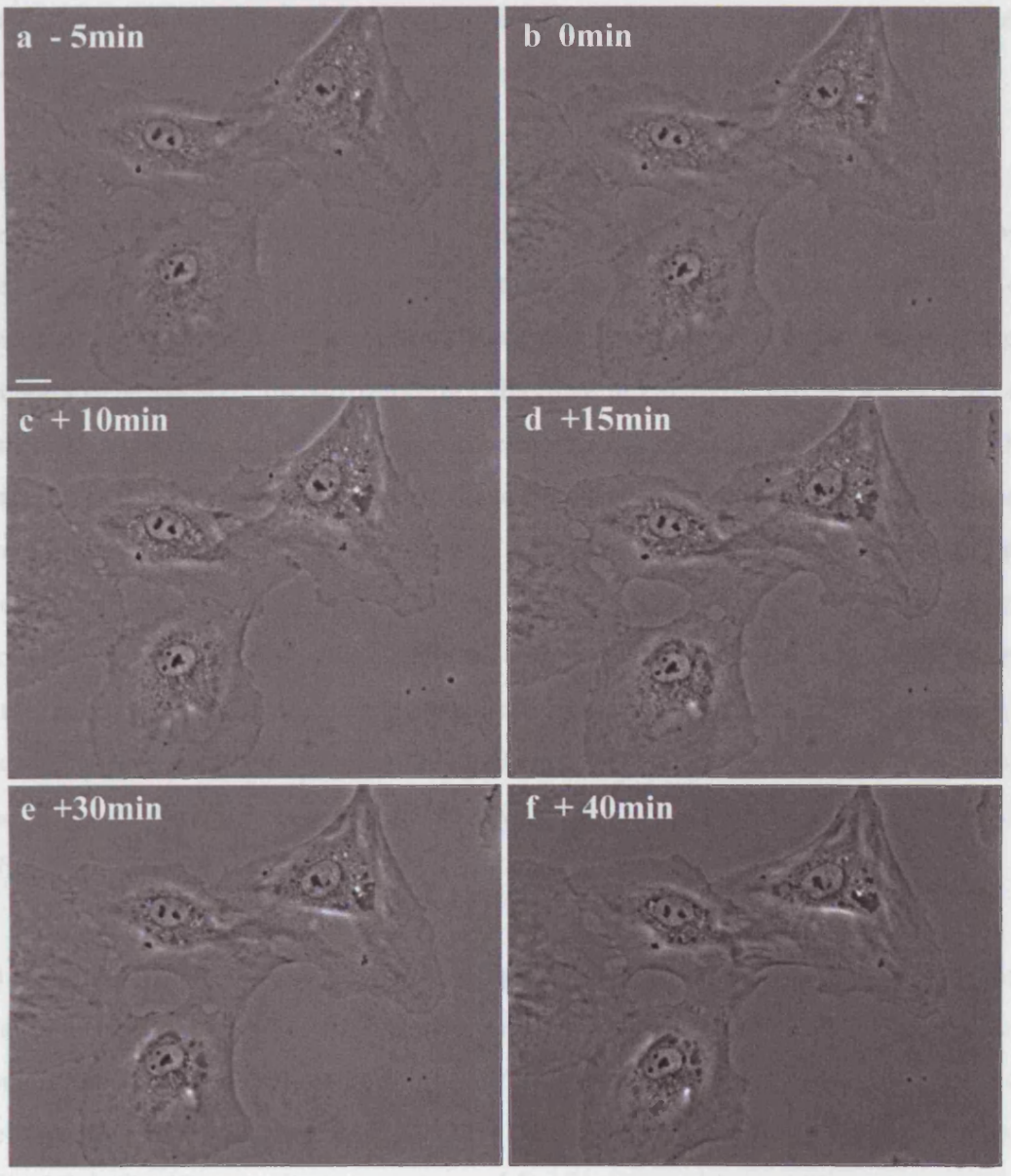
To confirm that the repulsive response I observe is due to ephrin-B2 stimualtion by EphB4 receptors, HUAECs were stimulated with pre-clustered Fc molecules. This is the Fc portion of IgG alone, and allows a duplicate assay to be carried out in the absence of the EphB4 moiety. Upon addition of pre-clustered Fc no retraction or membrane blebbing was observed (Fig. 4.5; Supplementary Material: Movie 4.3). Even after 40min stimulation cells remained well spread and continued to ruffle (Fig. 4.5f).

4.2.3 Analysis of morphological changes seen in response to stimulation of HUAECs with pre-clustered EphB4-Fc

I have shown using phase time-lapse microscopy that HUAECs undergo a repulsive cell retraction response to treatment with soluble pre-clustered EphB4-Fc (4.2.2). In order to analyse the cellular changes that take place after pre-clustered EphB4-Fc treatment, cells were fixed and stained with the cell membrane marker Concanavalin A (Con A) to investigate cell shape changes, and with fluorescently tagged phalloidin to look at actin cytoskeletal rearrangements.

Con A is a lectin that binds to carbohydrate moieties on the surface of cells (specifically A-linked mannose). HUAECs, fixed with formaldehyde, were treated with biotinylated succinylated Con A (20μg/ml), which binds to the cell surface and can then be detected using the vectastain system (see 2.3.1). This method highlights clearly the retraction fibres left behind after cell rounding, allowing quantification of the extent of retraction taken place. As shown in Fig. 4.6 and seen from the time-lapse studies, (Fig. 4.3 & 4.4 together with movies 4.1 & 4.2), unstimulated HUAECs are flat with spread lamellae (Fig. 4.6a). After 10min pre-clustered EphB4-

Figure 4.5 HUAECs stimulated with pre-clustered Fc do not retract



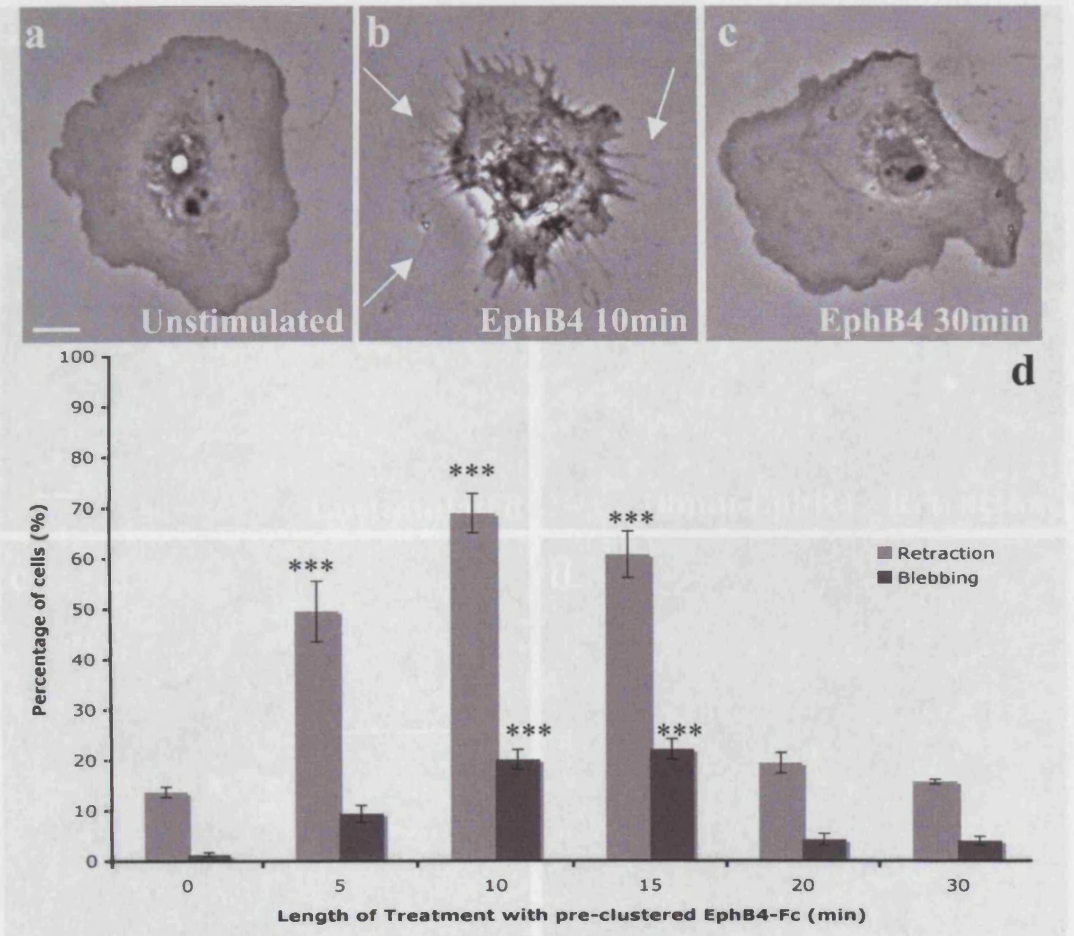
Phase time-lapse stills showing HUAECs treated with pre-clustered Fc alone (5μg/ml). Unstimulated cells are well-spread and ruffling (a). Upon stimulation cells remained well-spread (b), continued to ruffle and showed no signs of retraction even after 40min stimulation (c-f). Scale bar 15μm. (See Supplementary Material: Movie 4.3).

Fc stimulation dramatic cell retraction occurs, characterised by the appearance of long retraction fibres and withdrawal of cell margins (Fig. 4.6 arrows in b). After 30min stimulation with pre-clustered EphB4-Fc, cells recover, re-form lamellae and re-spread (Fig. 4.6c).

Quantification of the response reveals that the majority of cells undergo retraction in response to EphB4-Fc stimulation (Fig. 4.9d). Retracting cells were counted as those displaying long retraction fibres or membrane blebbing. A small proportion of unstimulated cells exhibited retraction but this could be due to endogenous Eph receptor expression by the surrounding cells (data not shown) causing a low level of ephrin-B signalling to occur. Cell retraction peaks after 10min with >65% cells retracting ($P < 0.001$) and >20% of these blebbing ($P < 0.001$; Fig. 4.6d). Phase time-lapse studies show that the timing of cell retraction and recovery, together with the extent of the response, can vary from cell to cell. This could be due to varying expression levels of ephrin-B2. Cells with a higher ephrin-B2 expression may exhibit a more dramatic response. It is known from immunocytochemistry that expression varies between cells and across a cell monolayer (data not shown). After 20-30min the extent of retraction has reduced to levels similar to those in unstimulated cells (Fig. 4.6d).

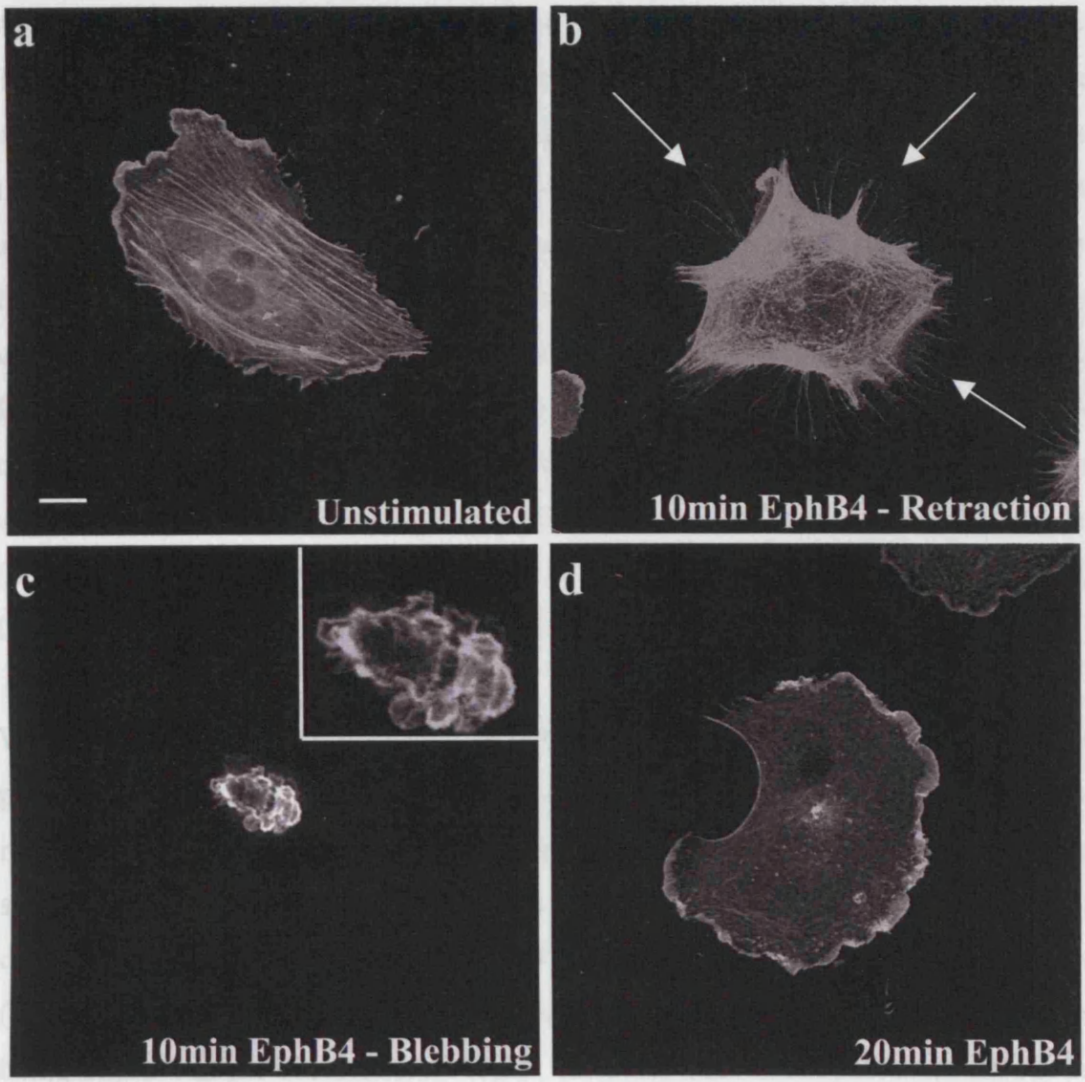
In order to further characterise the morphological changes taking place, F-actin staining using fluorescently labelled phalloidin was used, which allows a detailed look at the actin cytoskeleton throughout the time course of retraction. Unstimulated cells display actin-rich membrane ruffles and contain numerous actin stress fibres (Fig.4.7a). By 10min cells have retracted leaving behind long actin-rich retraction fibres (Fig. 4.7 arrows in b), and a proportion of cells undergo the more exaggerated response of membrane blebbing (Fig. 4.7c). As the cells retract there appears to be a re-arrangement of actin stress fibres (Fig. 4.7b). By 20min the majority of cells have recovered, displaying large lamellae and strikingly very few, if any, actin stress fibres (Fig. 4.7d). A rearrangement of the actin cytoskeleton occurs as a consequence of EphB4-Fc triggered ephrin-B2 signalling.

Figure 4.6 Quantification of retraction and blebbing after stimulation of HUAECs with pre-clustered EphB4-Fc



Staining for Concanavalin A highlights the dramatic retraction seen upon treatment with pre-clustered EphB4-Fc (5μg/ml). Unstimulated cells are well-spread (a). After 10min EphB4-Fc stimulation retraction was observed, characterised by the appearance of long retraction fibres (arrows in b), and by 30min the cells have re-spread (c). Quantification shows that a transient increase in both retraction (ANOVA $P < 0.0001$) and blebbing (ANOVA $P < 0.0001$) occurs over time. After 10min pre-clustered EphB4-Fc stimulation, >65% of cells had retracted ($P < 0.001$; d) and 20% of these exhibited membrane blebbing ($P < 0.001$; d). The cells quickly recover from these shape changes. By 20min post-stimulation retraction and blebbing have reduced to near control levels (d). The experiment was repeated >10 times with a minimum of 1000 cells counted in total per time point. (Asterisks denote statistical difference compared with unstimulated cells). Scale bar 15μm.

Figure 4.7 Actin changes seen upon treatment of HUAECs with EphB4



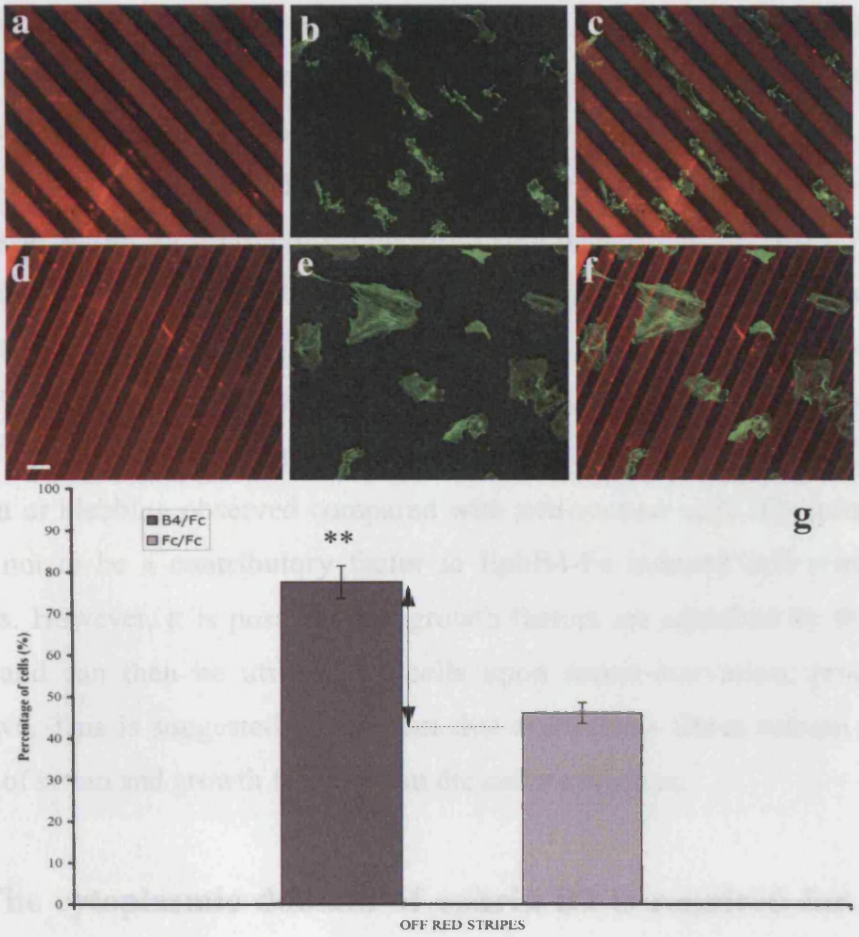
HUAECs were stained for F-actin with TRITC-phalloidin. Unstimulated HUAECs contain numerous actin stress fibres and are well-spread with ruffling lamella (a). After stimulation with pre-clustered EphB4-Fc (5μg/ml) for 10min cells retract (b) leaving behind actin-rich retraction fibres (arrows in b). Blebbing occurred in >20% of cells (c). Despite the continued presence of EphB4-Fc these shape changes were rapidly reversed. After 20min the majority of cells are well-spread, but strikingly lack actin stress fibres (d). Scale bar 15μm.

4.2.6 Substrate bound EphB4 is repulsive to ephrin-B2 expressing HUAECs

As stated previously both Eph receptors and ephrins are membrane-tethered proteins. Therefore in order for signalling to occur *in vivo,* cell-cell contact is crucial. One assay that replicates this more fully than stimulation with soluble receptor bodies is the stripe assay. Pre-clustered EphB4-Fc was immobilised on coverslips in alternating stripes consisting of pre-clustered EphB4-Fc versus Fc alone (EphB4/Fc). HUAECs were then plated onto these EphB4/Fc striped coverslips, thus encountering the receptor in a more physiologically relevant manner. As a control, HUAECs were also plated onto coverslips patterned with alternating stripes of pre-clustered Fc alone (Fc/Fc). After 24h the cultures were fixed and migrating HUAECs were detected using FITC-conjugated phalloidin to detect F-actin (green). EphB4-Fc (or Fc in the control) was localised with antibodies that recognise the anti-human IgG used for clustering (visualising the red stripes).

When offered the choice between EphB4-Fc versus Fc alone, HUAECs exhibit a preference for migration on the black, non-receptor stripes (Fig. 4.8a-c), displaying a repulsive response to substrate bound EphB4. 77.6% of cells migrated onto the Fc stripes with only 22.4% of cells on the EphB4 stripes (P <0.01; Fig. 4.8g). If distribution of cells is truly random, 55% of cells would lie on the red stripes (EphB4-Fc) and 45% on the black stripes (Fc), due to the width of the red stripes being 50μm and the black stripes 40μm. In the control situation where HUAECs were plated onto substrata consisting of alternating stripes of pre-clustered Fc, HUAECs showed no preference in their migratory behaviour (Fig. 4.8d-f). 53.9% of cells adhered to the wider red stripes and 46.1% to the black stripes as expected (Fig. 4.8g). It is also noted that cells plated onto the EphB4-Fc/Fc patterned coverslip undergo morphological changes. The cells no longer appear flat and well-spread (Fig. 4.8e & green in f), but become elongated in appearance (Fig. 4.8b & green in c). This allows the cells to lie in between the EphB4-Fc stripes (Fig. 4.8c). These findings corroborate the repulsive retraction response seen in response to soluble pre-clustered EphB4-Fc stimulation during phase time-lapse microscopy, and by immunocytochemistry.

Figure 4.8 HUAECs display a repulsive response to substrate-bound EphB4-Fc



HUAECs were cultured on alternating stripes of pre-clustered EphB4-Fc/Fc versus Fc/Fc. Stripes were visualised by detection of the IgG used for clustering (red). HUAECs were visualised by FITC-phalloidin used to detect F- (green). HUAECs avoid stripes containing EphB4-Fc and become elongated in appearance (b & c). Cells migrate randomly over the Fc stripes and remained well-spread (e & f). If distribution was random 55% of cells would lie on red stripes and 45% off, due to the slightly larger width of red stripes. This was true for cells on the Fc striped coverslip (g). However, >75% of cells avoided the EphB4-Fc stripes ($P < 0.01$; g). The experiment was repeated 3 times and a minimum of 200 cells counted per experiment. Scale bar 62.5μm.

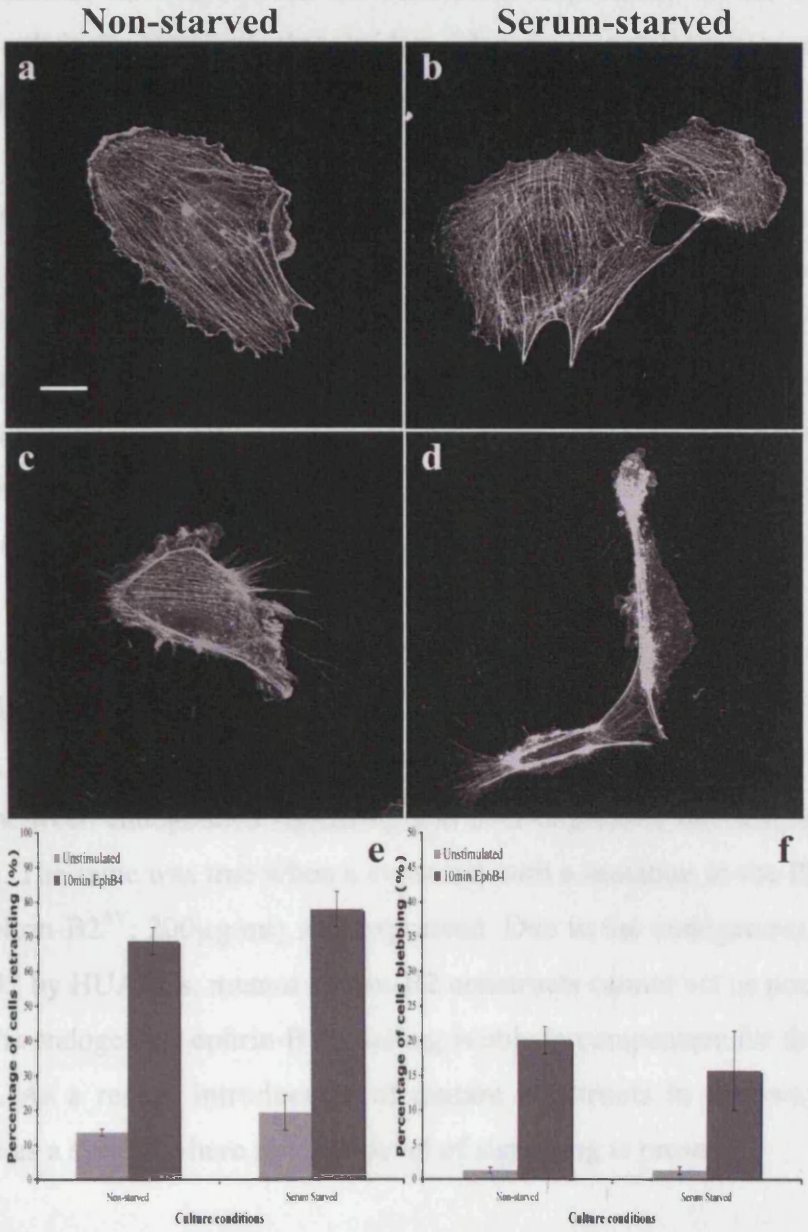
4.2.7 Removal of serum factors does not affect cell retraction triggered by EphB4

In chapter 3, I observed that Swiss 3T3 cells exogenously expressing ephrin-B2 undergo a loss of cell-cell contact response to EphB receptor stimulation. This effect was shown to be dependent on the presence of serum factors or FGF. In order to determine if HUAEC retraction requires serum factors, cells were depleted of serum and growth factors for 16h before EphB4-Fc stimulation. Unlike Swiss 3T3 cells, which lose their actin stress fibres upon starvation, HUAECs exhibited no obvious changes to their morphology or actin cytoskeleton (Fig. 4.9a&b). Upon pre-clustered EphB4-Fc stimulation, cells retracted their margins and exhibited long retraction fibres (Fig. 4.9c&d). Serum-starvation resulted in no difference in the levels of retraction or blebbing observed compared with non-starved cells. Therefore serum appears not to be a contributory factor to EphB4-Fc induced cell retraction in HUAECs. However, it is possible that growth factors are adsorbed by the gelatin matrix, and can then be utilised by cells upon serum-starvation, rendering it ineffective. This is suggested by the fact that actin-stress fibres remain after the removal of serum and growth factors from the culture medium.

4.2.8 The cytoplasmic domain of ephrin-B2 is required for EphB4 triggered retraction in HUAECS, but Grb4 is not required

In order to attempt to determine the mechanism of signalling underlying EphB4 triggered cell retraction, I have expressed various ephrin-B2 mutant constructs by microinjection. The effects of these mutations were then analysed and a clearer picture of the signalling domains necessary for retraction obtained. To confirm that microinjection did not result in adverse effects for the injected HUAECs, empty vector alone (pRK5; 200μg/ml), plus biotin dextran (2mg/ml) as an injection marker, was microinjected into sub-confluent cells. As expected, a normal retraction response was seen, with just over 60% pRK5-injected cells retracting (Fig. 4.10d). Over-expression of full-length ephrin-B2 (200μg/ml) prior to treatment with pre-clustered EphB4-Fc caused a dramatic shift towards cell rounding and produced an

Figure 4.9 The effect of serum starvation on EphB4-Fc induced cell retraction and membrane blebbing



F-actin was detected using TRITC-phalloidin. Unstimulated HUAECs are well-spread, flat cells rich in actin stress fibres (a). Serum-starvation does not affect cell morphology and cells retained their actin stress fibres (b). Serum-starvation did not affect the retraction response to EphB4-Fc stimulation (b-f). The levels of retraction and blebbing observed under serum-starved conditions were not different to those exhibited by non-starved cells (e & f). The experiment was repeated 3 times with a minimum of 300 cells counted in total per condition. Scale bar 15μm.

exaggerated cell retraction response (Fig. 4.10a-c), with 91% of cells retracting compared to only 62% of pRK5-injected cells ($P < 0.01$; Fig. 4.10d).

Numerous studies have described the functional importance of the cytoplasmic domain of ephrin-Bs, in particular the last 33 amino acids (Adams et al., 2001; Cowan and Henkemeyer, 2001; Tanaka et al., 2005; Xu et al., 2000). Truncated ephrin-B2, (comprising residues 1-253), lacks the entire intracellular domain including the functionally important tyrosine residues (Bong et al., 2004; Kalo et al., 2001; Su et al., 2004), and the PDZ binding domain. Expression of truncated ephrin-B2 (200μg/ml) in HUAECs, prior to pre-clustered EphB4-Fc stimulation, triggered a weaker retraction response than over-expression of full-length ephrin-B2 ($P <$ 0.001), equal to that observed for pRK5-injected cells (Fig. 4.10g-h). This suggests that the ephrin-B2 cytoplasmic domain is necessary for retraction, since the enhanced response seen after over-expression of full-length ephrin-B2 does not occur.

Expression of a mutant construct where all 5 functionally important tyrosine phosphorylation sites have been mutated to phenylalanine (5Y-F ephrin-B2; 200μg/ml), prior to pre-clustered EphB4-Fc stimulation, triggered a level of retraction between endogenous signalling and over-expressed full-length ephrin-B2 (Fig 4.10g). The same was true when a construct with a mutation in the PDZ binding domain (ephrin-B2$^{\Delta V}$; 200μg/ml) was expressed. Due to the endogenous expression of ephrin-B2 by HUAECs, mutant ephrin-B2 constructs cannot act as non-functional proteins. The endogenous ephrin-B signalling is able to compensate for the mutations introduced. As a result, introduction of mutant constructs in this way is not as informative as a system where no basal level of signalling is present.

The SH2/SH3 domain adaptor protein Grb4 has been shown to bind to ephrin-B1 in a phosphotyrosine dependent manner and initiate actin cytoskeletal changes (Cowan and Henkemeyer, 2001). I have shown that the loss of cell-cell contact response seen in Swiss 3T3 cells, upon EphB2 stimulation of exogenous ephrin-B2, is Grb4 dependent (see chapter 3). In order to discover whether the retraction response seen upon initiation of ephrin-B2 signalling in HUAECs is also Grb4 dependent, a dominant negative Grb4 construct was expressed in HUAECs prior to pre-clustered EphB4-Fc stimulation. Unlike the mutant ephrin-B2 constructs introduced via

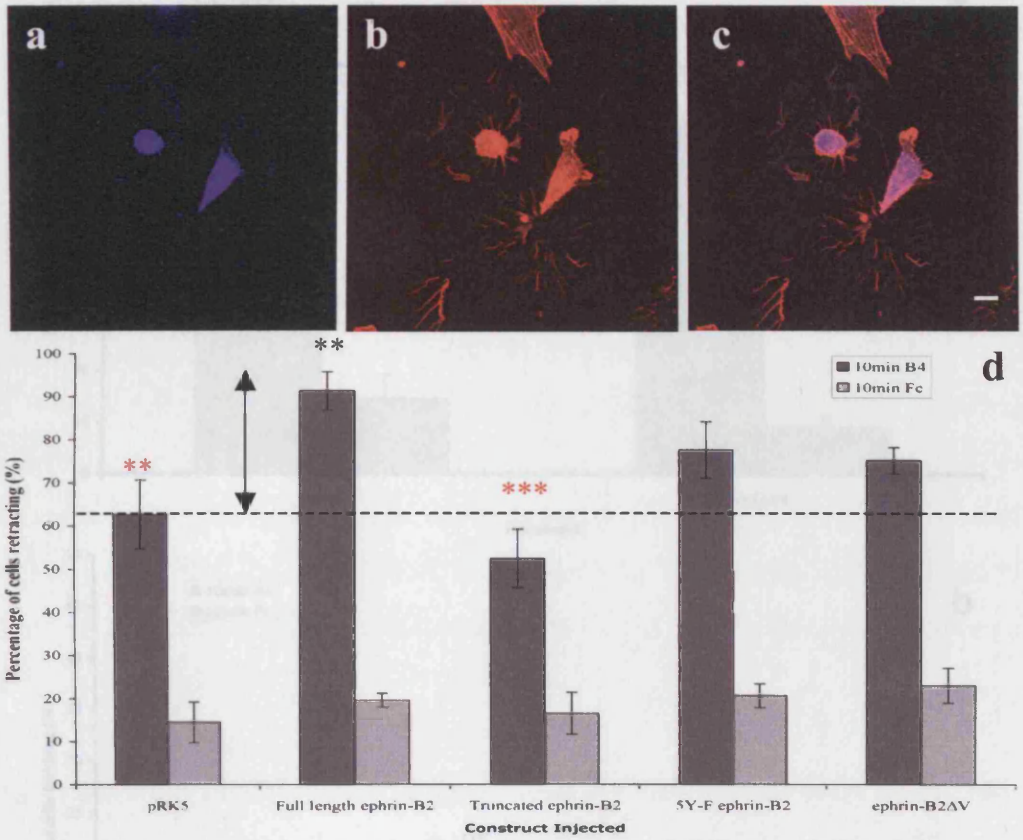
microinjection, this construct can act as a dominant negative. The construct comprises the Grb4 SH2 domain alone and therefore retains the ability to bind to endogenous ephrin-Bs but is unable to initiate downstream signalling events due to the lack of SH3 domains, which are known binding sites for many downstream signalling molecules. Expression of dnGrb4 (200μg/ml) resulted in no inhibition of either retraction or blebbing compared with control injection of pRK5 (Fig. 4.11a&b). Therefore, unlike the loss of cell-cell contact observed in response to clutsering of exogenous ephrin-B2 in Swiss 3T3 cells, Grb4 is not necessary for retraction and blebbing as a result of ephrin-B2 signalling in HUAECs, suggesting that a different signalling pathway is used to generate this repulsive response.

Cells microinjected with ephrin-B2 or other mutant constructs were also stimulated with pre-clustered Fc for 10min to confirm that the effects seen were specific to EphB4 stimulation. No difference in the level of retraction was observed regardless of the construct injected (Fig. 4.10d&e; Fig. 4.11).

4.3 Discussion

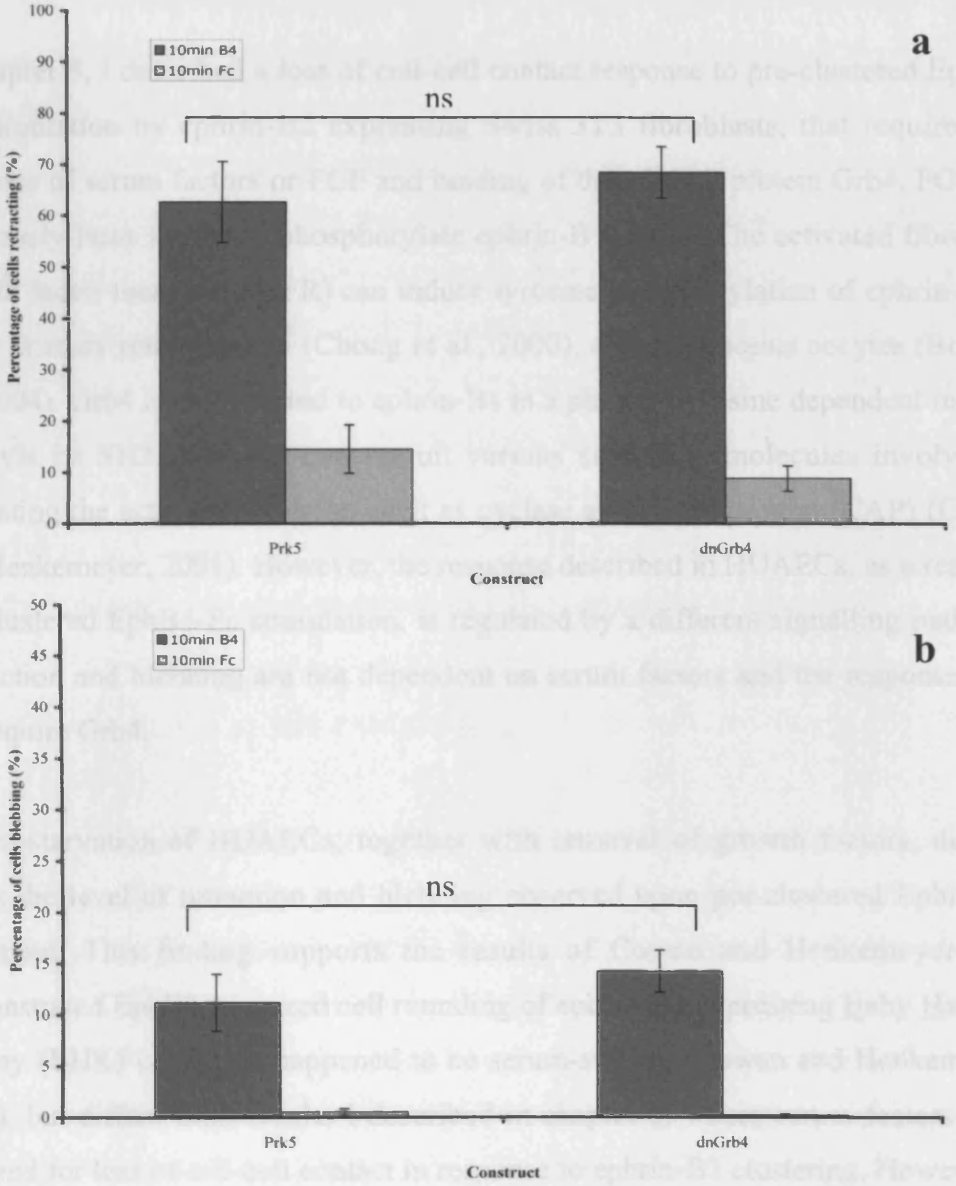
In this chapter, I have described the repulsive response of HUAECs to ephrin-B2 signalling after both soluble Eph receptor treatment and interaction with substrate bound Eph receptor. Stimulation of sub-confluent HUAECs in culture, which endogenously express ephrin-Bs, with pre-clustered EphB4-Fc, results in rapid cell retraction characterised by the withdrawal of cell margins, and the appearance of long actin-rich retraction fibres; a proportion of the cells round up and undergo membrane blebbing (Figs 4.6 & 4.7). Retraction peaks 10min after Eph receptor addition (Fig. 4.6). The response is transient, the majority of cells having recovered and re-spread lamellae within 30min (Fig. 4.6), concomitant with a loss of actin stress fibres (Fig. 4.7). The retraction response occurs independently of serum factors, (Fig. 4.9), and Grb4, (Fig. 4.11).

Figure 4.10 The importance of the cytoplasmic domain of ephrin-B2 for EphB4 triggered cell retraction



HUAECs were microinjected with various mutant ephrin-B2 constructs (200μg/ml) together with biotin dextran (2mg/ml) as an injection marker (blue), and the effect on the EphB4-triggered retraction response was investigated. F-actin was detected using TRITC-phalloidin (red). Expression of pRK5 alone resulted in 61% cells retracting (d). Over-expression of full-length ephrin-B2 caused a dramatic shift in the response towards cell rounding (a-c) and an enhanced retraction response, with 91% injected cells retracted ($P < 0.01$; d). Truncated ephrin-B2, lacking the entire cytoplasmic domain did not trigger an increased retraction response (d). Over-expression of 5Y-F ephrin-B2, where all the tyrosine residues have been mutated to phenylalanine and ephrin-B2 with a mutation in the PDZ binding domain (ephrin-B2ΔV) did not cause an inhibition of retraction (d). No retraction above control levels was observed upon stimulation of expressed constructs with pre-clustered Fc for 10min (d). Each experiment was carried out a minimum of 5 times with at least 250 cells counted in total per condition. (Red asterisks denote statistical significance compared to full-length ephrin-B2. Black asterisks refer to pRK5-injected cells). Scale bar 15μm.

Figure 4.11 Grb4 is not required for the retraction response



HUAECs were microinjected with a dominant negative Grb4 construct (200µg/ml), comprising the isolated SH2 domain of Grb4, plus biotin dextran (2mg/ml). Upon stimulation with pre-clustered EphB4-Fc (5µg/ml) for 10min no difference in the level of retraction (a) and blebbing (b) was observed, compared with control injections of pRK5 (200µg/ml) plus biotin dextran (2mg/ml). Cells expressing dnGrb4 stimulated with pre-clustered Fc for 10min do not exhibit retraction or blebbing (a&b). The experiment was carried out 5 times with a minimum of 250 cells counted in total per condition.

4.3.1 Ephrin-B2 signalling triggers a cell retraction response in HUAECs independently of serum factors and Grb4

In chapter 3, I described a loss of cell-cell contact response to pre-clustered EphB2-Fc stimulation by ephrin-B2 expressing Swiss 3T3 fibroblasts, that required the presence of serum factors or FGF and binding of the adaptor protein Grb4. FGF has previously been shown to phosphorylate ephrin-B ligands. The activated fibroblast growth factor receptor (FGFR) can induce tyrosine phosphorylation of ephrin-Bs in chick primary retina *in vivo* (Chong et al., 2000), and in *Xenopus* oocytes (Bong et al., 2004). Grb4 is able to bind to ephrin-Bs in a phosphotyrosine dependent manner and, via its SH3 domains, can recruit various signalling molecules involved in regulating the actin cytoskeleton such as cyclase associated protein (CAP) (Cowan and Henkemeyer, 2001). However, the response described in HUAECs, as a result of pre-clustered EphB4-Fc stimulation, is regulated by a different signalling pathway. Retraction and blebbing are not dependent on serum factors and the response does not require Grb4.

Serum-starvation of HUAECs, together with removal of growth factors, did not affect the level of retraction and blebbing observed upon pre-clustered EphB4-Fc treatment. This finding supports the results of Cowan and Henkemeyer who demonstrated EphB2-triggered cell rounding of ephrin-B1 expressing Baby Hamster Kidney (BHK) cells, that happened to be serum-starved (Cowan and Henkemeyer, 2001), but differs from results I described in chapter 3, where serum factors were required for loss of cell-cell contact in response to ephrin-B2 clustering. However, it should be noted that unlike Swiss 3T3 cells, that lose actin-stress fibres upon serum depletion, this is not the case for HUAECs or BHKs, indicating they may not be truly growth factor free. HUAECs are plated onto a gelatin substrate and this may bind serum factors (growth factors, serum lipids) that may remain available after serum-starvation, rendering it less effective.

The results presented in this chapter describe a repulsive retraction response to ephrin-B2 signalling, that is not dependent on Grb4, as demonstrated by the failure of dominant negative Grb4 to inhibit cell retraction. However, this finding does not

corroborate those of Cowan and Henkmeyer, who describe cell rounding in response to pre-clustered EphB2-Fc stimulation of ephrin-B1 expressing BHK cells, which is dependent on Grb4 binding to ephrin-B1 in a phosphotyrosine dependent manner (Cowan and Henkemeyer, 2001). In addition, the lack of a requirement for Grb4 does not corroborate the results presented in chapter 3, describing a loss of cell-cell contact response to ephrin-B2 clustering in Swiss 3T3 fibroblasts that is dependent on Grb4. However, In 2003 Xu and colleagues highlighted another example of cell rounding in response to ephrin-B1 activation. Over-expression of ephrin-B1 in HEK 293 cells led to cell rounding which occurred independently of Grb4 binding (Xu et al., 2003) and would therefore agree with the Grb4 independent cell retraction phenotype observed in HUAECs. Interestingly, data from the Nobes laboratory has recently shown Grb4 to be important for the re-spreading event that follows cell retraction in response to ephrin-B2 signalling. However, the importance of Grb4 in order that cells can recover to re-spread lamellae remains to be determined.

4.3.2 Development of the vasculature

Genetic experiments carried out by many groups have unequivocally demonstrated the importance of bi-directional Eph/ephrin signalling during vascular development (reviewed in Davy and Soriano, 2005). However, such studies have provided limited information about the cellular consequences of activation of EphB receptors and ephrin-B ligands. The findings at this stage appear contradictory. EphB receptor activation has been demonstrated to increase the migration, adhesion and proliferation of endothelial cells (Adams et al., 1999; Maekawa et al., 2003; Stein et al., 1998b; Steinle et al., 2002), and can promote endothelial sprouting (Adams et al., 1999; Stein et al., 1998b). In contrast activation of EphBs has also been shown to inhibit endothelial cell migration, proliferation and sprouting (Fuller et al., 2003; Hamada et al., 2003; Kim et al., 2002; Sturz et al., 2004). Similarly, ephrin-B2 activation has been shown to increase endothelial cell migration, adhesion, sprouting and proliferation (Fuller et al., 2003; Hamada et al., 2003; Huynh-Do et al., 2002; Steinle et al., 2003), but also to decrease sprouting activity (Zhang et al., 2001). The reason for the observed differences in response is not known. It could be due to the different cell types used or, alternatively, a result of differing experimental

conditions. For example, some studies investigated the effect on VEGF induced endothelial migration (Kim et al., 2002; Sturz et al., 2004), whereas in other systems the receptor/ligand alone was sufficient (Steinle et al., 2003).

The findings from this study would support a model for both endothelial cell repulsion and attraction in response to ephrin-B2 signalling. Cycles of endothelial cell retraction and recovery may be necessary for the correct migration of cells throughout blood vessel development and the specification of arteries and veins. However, more work is needed in order to investigate this further.

4.3.3 Significance of membrane blebbing

In this chapter, I have demonstrated that a subset of HUAECs can undergo membrane blebbing as a consequence of ephrin-B2 signalling. Membrane blebbing is defined as the protrusion and subsequent retraction of plasma membrane bound blebs on the surface of cells and is a morphological change characteristic of apoptotic cells. During apoptosis cells undergo a loss of matrix attachment and retraction followed by plasma-membrane blebbing, which is usually transient. Cell fragmentation and death then follow (Vermeulen et al., 2005). The Rho GTPase target ROCK I has been shown to be cleaved by caspases during apoptosis (Coleman et al., 2001) and ROCK I is known to stimulate actomyosin-based contractility. ROCK can activate myosin light chain kinase indirectly, by phosphorylating the myosin binding subunit of myosin light chain phosphatase. This renders it inactive and therefore prevents dephosphorylation of myosin light chain allowing actomyosin contractility (Amano et al., 1996; Kimura et al., 1996). In addition, ROCK can act directly, by phosphorylating myosin light chain kinase itself in some systems (Amano et al., 1996).

Membrane blebbing in this instance, displayed in a proportion of HUAECs stimulated with pre-clustered EphB4-Fc, is not a sign of apoptosis. ROCK I is not cleaved (data not shown), and the cells are not dying, they rapidly recover to re-spread lamellae, as demonstrated by phase time-lapse microscopy (Supplementary Material; Movie 4.1 & 4.2). Membrane blebbing could simply be a more dramatic

retraction response. Those cells that undergo membrane blebbing may express higher levels of ephrin-Bs than those that simply retract their margins. Membrane blebbing is not a phenomenon restricted to apoptotic cells. Blebs have been observed at the leading edge of migrating cells, playing a role in cell locomotion (Grinnell, 1982; Keller et al., 1985; Trinkaus, 1973; Trinkaus, 1980) and blebbing has been seen to precede cell spreading after plating cells in tissue culture dishes (Bereiter-Hahn et al., 1990; Cunningham, 1995; Hoglund, 1985).

Since ROCK has a role to play in actomyosin contractility, and the retraction/blebbing response to ephrin-B2 signalling followed by re-spreading is reminiscent of a contraction event, the role of ROCK in cell retraction as a result of ephrin-B2 signalling in HUAECs will be investigated in chapter 5.

4.3.4 The cytoplasmic domain of ephrin-B2 is implicated in retraction triggered as a result of ephrin-B2 activation

Microinjection experiments introducing mutant ephrin-B2 constructs into HUAECs have proved to have a limited use. Swiss 3T3 fibroblasts, which do not express ephrin-B2, can be microinjected with mutant constructs to determine the effect of specific mutations on the loss of cell-cell contact observed as a result of exogenous ephrin-B2 clustering. This is not the case in HUAECs. Endogenous ephrin-B2 triggers a retraction response that is not blocked by over-expression of truncated or mutant ephrin-B2 constructs. However, an exaggerated response was observed upon injection of full-length ephrin-B2, with an increased number of cells responding and a much higher incidence of cell rounding. A requirement for the cytoplasmic domain of ephrin-B2 for these responses is therefore suggested by the reduction of retraction down to below endogenous levels, upon stimulation of truncated ephrin-B2, which lacks the entire intracellular domain.

Phosphorylation on tyrosine residues was the first reported indication of reverse signalling (Bruckner et al., 1997) and ephrin-Bs have been shown to become tyrosine phosphorylated in numerous studies (Cowan and Henkemeyer, 2001; Palmer et al., 2002; Tanaka et al., 2005). However, expression of 5Y-F ephrin-B2, in which all five

potential tyrosine phosphorylation sites in the carboxy-terminal tail have been mutated to phenylalanine, prior to pre-clustered EphB4-Fc stimulation, triggers a level of retraction in between that of full-length ephrin-B2 stimulation and endogenous signalling. A range of PDZ domain protein interactions with ephrin-Bs are known to occur (Bruckner et al., 1999; Lin et al., 1999; Lu et al., 2001; Palmer et al., 2002; Torres et al., 1998). For example, PDZ-RGS3 has been shown to be essential for ephrin-B mediated neuronal cell migration *in vitro* (Lu et al., 2001). *In vivo* the PDZ interaction site has recently been shown to be required for remodelling of the lymphatic vasculature (Makinen et al., 2005) and also for midline fusion events (Davy et al., 2004). PICK-1, PDZ-RGS3 and Dvl2 are all expressed in the lymphatic endothelium, and the localisation patterns of PDZ-RGS3 and Dvl2 were altered in mutant mice homozygous for a mutation in the C-terminal valine of the PDZ binding motif (ephrin-B2$^{\Delta V/\Delta V}$) (Makinen et al., 2005) suggesting a direct interaction and a function as ephrin-B2 effectors in this tissue. In HUAECs over-expression of ephrin-B2$^{\Delta V}$ resulted in a level of retraction midway between that seen upon stimulation of expressed full-length ephrin-B2 and endogenous signalling. The results from both 5Y-F ephrin-B2 and ephrin-B2$^{\Delta V}$ over-expression were therefore inconclusive. Interestingly, the cell rounding response to ephrin-B1 activation described by Xu and colleagues was shown to be independent of tyrosine phosphorylation and PDZ domain protein interactions, requiring residues in the cytoplasmic domain other than the last 33 amino acids (Xu et al., 2003). This phenotype was also independent of Grb4. Since EphB4 triggered retraction in HUAECs is independent of Grb4 it may suggest that the signalling pathway utilised here is also independent of tyrosine phosphorylation and PDZ domain protein interactions.

Src family kinases have been shown to be involved in tyrosine phosphorylation of ephrin-Bs (Palmer et al., 2002). In an attempt to determine whether a requirement for tyrosine phosphorylation does exist, the involvement of Src family kinases in the retraction response to ephrin-B2 signalling in HUAECs was investigated. Two pharmacological inhibitors were tested, PP2 and SU6656. However, results obtained from these experiments were inconclusive since pre-treatment caused cells to retract even in the control situation, without EphB4 stimulation.

4.3.5 Conclusions

HUAECs endogenously expressing ephrin-B2 undergo a repulsive response to stimulation with both soluble and substrate bound EphB4-Fc. Treatment of HUAECs with soluble EphB4-Fc triggers a dramatic retraction response and, in a subset of cells, membrane blebbing. The response is transient, cells re-spread lamellae within 30min, concomitant with a loss of actin stress fibres. Cell retraction and blebbing is independent of Grb4, since dominant negative Grb4 did not block the response.

Cell rounding as a consequence of ephrin-B1 activation has recently been demonstrated by two groups (Cowan and Henkemeyer, 2001; Xu et al., 2003). However, the signalling pathways mediating this response differ dramatically. Cell rounding can occur as a result of tyrosine phosphorylation-dependent Grb4 binding (Cowan and Henkemeyer, 2001), and also independently of tyrosine phosphorylation, PDZ domain protein interactions and Grb4, but requiring c-jun amino terminal kinase (JNK) activity (Xu et al., 2003).

The retraction response described in HUAECs does not require Grb4, is rapid, peaking after 10min pre-clustered EphB4-Fc stimulation, and transient with cells having re-spread their lamellae within 30min. In BHKs cell rounding and loss of actin stress fibres was observed much later, after 6h pre-clustered EphB2-Fc stimulation, and the response was not transient (Cowan and Henkemeyer, 2001). However, in HUAECs endogenous protein is activated, but in BHKs ephrin-B1 was expressed by transfection. It is also possible that the responses could be cell-type dependent. Interstingly Cowan and Henkemeyer did report a loss of actin stress fibres concomitant with cell rounding and this finding is corroborated upon re-spreading of EphB4 stimulated HUAECs.

The data presented in this chapter do corroborate that of Xu and colleagues, where ephrin-B1 activation resulted in cell rounding independently of Grb4 as is the case here. In that study, JNK activity was found to be crucial for cell rounding (Xu et al., 2003). In this study, cell rounding occurs as a result of ephrin-B2 signalling and a potential role for JNK in HUAEC retraction as a result of EphB4 stimulation will be

investigated in chapter 5. In addition, since the small GTPase Rho is known to regulate actin stress fibre formation, and a loss of actin stress fibres is observed upon re-spreading, I have investigated the involvement of Rho in the retraction and subsequent recovery of HUAECs in response to pre-clustered EphB4-Fc stimulation.

In Swiss 3T3 cells, exogenous ephrin-B2 clustering resulted in a Grb4 dependent loss of cell-cell contact response (see chapter 3) reminiscent of the cell rounding seen after ephrin-B1 activation in BHK cells (Cowan and Henkemeyer, 2001). However, stimulation of ephrin-B2 in HUAECs triggers a cell rounding event independently of Grb4, as described after ephrin-B1 activation in HEK 293 cells (Xu et al., 2003). These data highlight the fact that there are two distinct pathways in operation regulating ephrin-B mediated cell adhesion.

Chapter 5

Retraction and blebbing triggered by ephrin-B2 signalling is mediated by Rho, ROCK and JNK activity

5.1 Introduction

In chapter 4, I showed that stimulation of HUAECs, that endogenously express ephrin-Bs, with pre-clustered EphB4-Fc, triggered a dramatic cell retraction event characterised by cell rounding, the appearance of long retraction fibres and, in a proportion of cells, membrane blebbing. Retraction is not dependent on the presence of serum factors or Grb4, suggesting that it is not dependent on tyrosine phosphorylation of ephrin-B2, but requires the intracellular domain of the ligand.

Numerous signalling proteins and adaptor molecules capable of interacting with ephrin-Bs have been identified (see 1.8), and the importance of reverse signalling into the ephrin expressing cell, has been demonstrated by *in vitro* and genetic studies. However, the underlying mechanism of ephrin-B activation, and the downstream signal transduction pathways involved remain unclear. The cell rounding and subsequent re-spreading response to ephrin-B2 signalling that I described in chapter 4 is reminiscent of a cell contraction event. Therefore, in an effort to elucidate further the signalling pathways downstream of ephrin-B2 in this retraction assay, a potential role for Rho family GTPases, the RhoA-associated kinase ROCK, and the MAP kinase c-Jun amino terminal kinase (JNK) were investigated.

Through its ability to promote both protrusion and contraction, the actin cytoskeleton is the driving force for cell migration. The molecular control of actin filament assembly and disassembly underlies cell motility, and the Rho family of small GTPases are particularly important for the actin reorganisations that take place during cell migration. Rho regulates stress fibre formation (Ridley and Hall, 1992), while Rac and Cdc42 regulate lamellipodia and filopodia respectively (Nobes and Hall, 1995; Ridley et al., 1992). Rho is known to induce stress fibre and focal adhesion formation by stimulating contractility and many cell types utilise contractile actin and myosin filaments to induce rapid, reversible changes in shape. A known down-stream target of Rho is the effector protein Rho kinase (ROCK). ROCK is known to stimulate actomyosin-based contractility by inducing the phosphorylation of the myosin regulatory light chain. This has been shown to occur both indirectly, via phosphorylation, and subsequent inactivation, of myosin light chain phosphatase

(Kimura et al., 1996), or directly, by phosphorylating myosin light chain kinase (MLCK) itself in some cell systems (Amano et al., 1996). Due to the rapid contraction response displayed by HUAECs in response to receptor stimulation, Rho and ROCK activity could be necessary for retraction to occur. In addition, the response is transient, followed by re-spreading, and upon recovery cells have lost their actin stress fibres, as described in chapter 4 (Fig. 4.7). Investigation into how ephrin-B ligands can modulate the actin cytoskeleton, potentially through the family of Rho GTPases, may shed light on the regulation of cell migration by ephrin-B reverse signalling.

A recent study highlighted a role for JNK activity in cell rounding induced by ephrin-B1 activation (Xu et al., 2003). In addition, earlier studies have shown JNK to be phosphorylated when both the forward (via Eph receptor) and reverse (via ephrin ligand) signalling pathways are switched on (Huynh-Do et al., 2002; Stein et al., 1998a). Accumulating evidence implicates the c-Jun amino terminal kinase (JNK) pathway in the regulation of cell migration, although the precise nature of JNK involvement, and the signalling pathways downstream of JNK activation are unclear. For example, JNK activation correlates with an increase in cell migration in several cell types (Abassi and Vuori, 2002; Hauck et al., 2001; Huynh-Do et al., 2002). In addition, the signalling molecules that activate JNK are essential for cell migration (Shin et al., 2001b; Xia et al., 2000; Yujiri et al., 2000), and inhibition of JNK impairs cell migration rates in several cell types, such as smooth muscle cells and Schwann cells (Kavurma and Khachigian, 2003; Yamauchi et al., 2003). JNK has also been shown to play a role in the migration of fibroblasts in wound healing assays (Javelaud et al., 2003), and in some aspects of cytoskeletal reorganisation in both Drosophila (Kaltschmidt et al., 2002; Martin-Blanco et al., 2000), and mammalian cells (Zhang et al., 2003). Also, several cytoskeleteton-associated proteins as well as adaptor proteins have recently been identified as JNK substrates, such as the actin-binding protein Spir (Otto et al., 2000) and the focal adhesion adaptor protein paxillin (Huang et al., 2003). Taken together these findings clearly implicate JNK in the control of cell migration in a broad range of cell types and in several developmental processes.

In this chapter, I have shown that EphB4 induced cell retraction in HUAECs is driven by actomyosin based contractility, and is dependent on both Rho and ROCK activity. JNK appears to be acting upstream of Rho-ROCK signalling. Interestingly, despite Rho being required for cell retraction to occur, Rho activity is dramatically down regulated at later times, coincident with a loss of actin-stress fibres seen during recovery and re-spreading of lamellipodia.

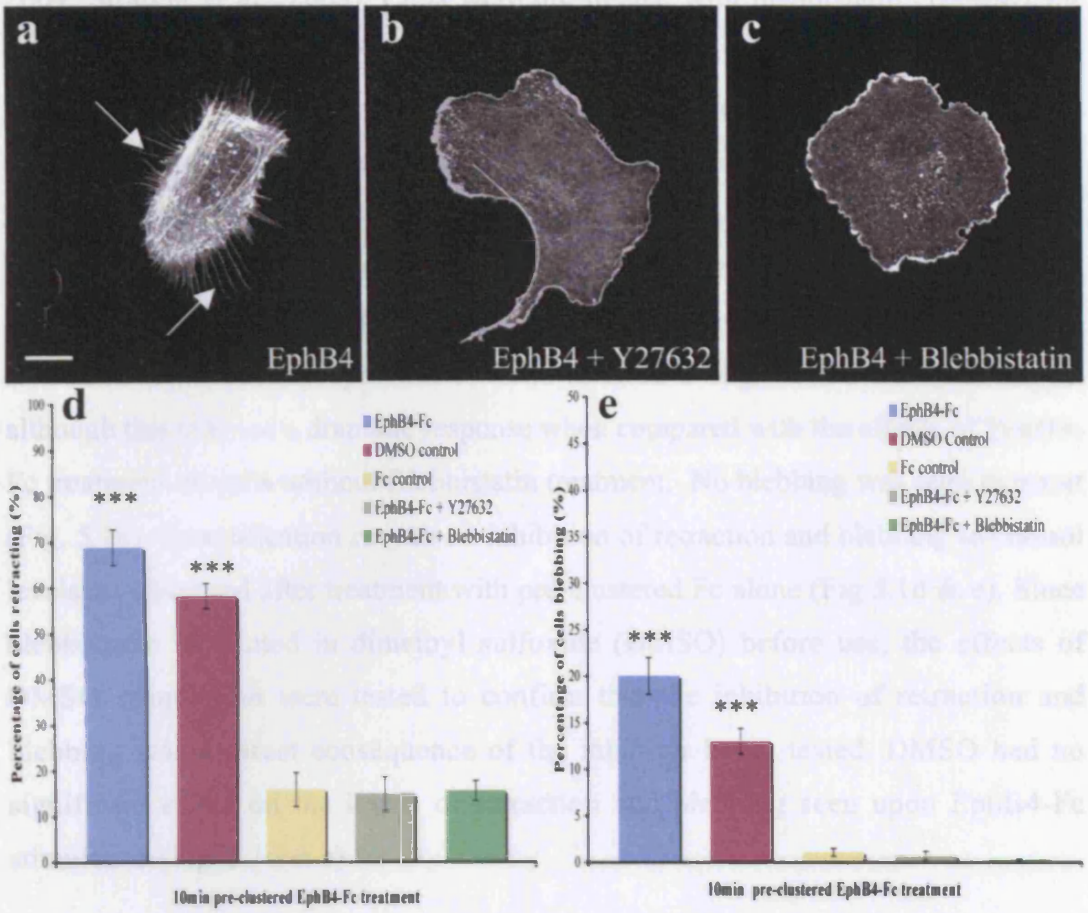
5.2 Results

5.2.1 Cell retraction induced by ephrin-B2 signalling is driven by acto-myosin contraction and dependent on Rho/ROCK signalling

The rapid cell retraction event I observe in response to pre-clustered EphB4-Fc stimulation of HUAECs could be driven by contraction of actin-myosin filaments leading to a cell contraction event. The Rho-ROCK pathway is known to be important for cell contractility to occur. Smooth muscle cell contraction and membrane blebbing during apoptosis are two examples of events mediated by the Rho-ROCK pathway (Coleman et al., 2001; Uehata et al., 1997). I therefore investigated a potential role for ROCK and acto-myosin contractility in this assay,

To determine the role of ROCK activity during the retraction response to ephrin-B2 activation, the effect of Y27632 on cell behaviour was investigated. Y27632 is a specific inhibitor of ROCK activity, with a similar IC_{50} for both ROCK isoforms (Ishizaki et al., 2000; Uehata et al., 1997). As described in chapter 4, cells stimulated with pre-clustered EphB4-Fc for 10min retracted and withdrew their cell margins ($P < 0.001$; Fig. 5.1a). Pre-treatment with the ROCK inhibitor Y27632 (20μM) for 30min resulted in a loss of actin stress fibres (Fig. 5.1b), and upon stimulation with pre-clustered EphB4-Fc, the cells remained well-spread and did not undergo retraction or blebbing compared to treatment with pre-clustered Fc alone (Fig 5.1b and d-e).

Figure 5.1 Cell retraction and blebbing triggered by ephrin-B2 activation is dependent on ROCK and actomyosin contractility



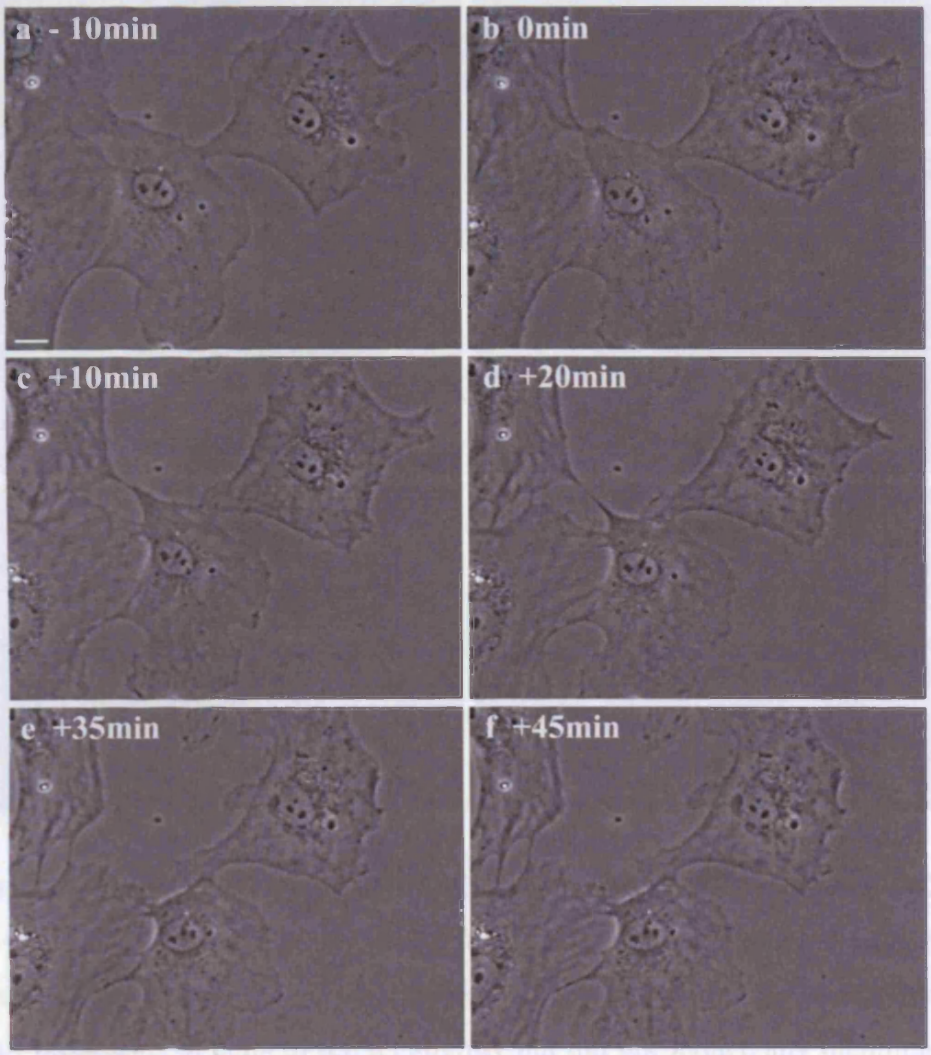
HUAECs were stained for F-actin using TRITC-phalloidin. Stimulation with pre-clustered EphB4-Fc (5μg/ml) for 10min resulted in dramatic cell retraction ($P < 0.001$; arrows in a). Pre-treatment with the Rho kinase inhibitor Y27632 (20μM; b) or the myosin II ATPase inhibitor blebbistatin (100μM; c), caused a loss of actin stress fibres, and the cells remain well-spread after pre-clustered EphB4-Fc stimulation (b & c). Quantification of the percentage of cells retracting (d) and blebbing (e) demonstrates that both Y27632 and blebbistatin, inhibit retraction and blebbing down to control levels (b-e). Treatment with Fc alone does not cause retraction (d) or blebbing (e). Pre-treatment with DMSO prior to EphB4-Fc stimulation had no significant effect with respect to retraction and blebbing (d & e). Asterisks denote statistical difference with respect to control Fc stimulation. Scale bar 15μm.

The same was true if cells were pre-treated with blebbistatin, a small molecule inhibitor of both muscle and non-muscle myosin II. This molecule inhibits the ATPase activity of myosin II and therefore actin-myosin contraction (Kovacs et al., 2004; Straight et al., 2003). Cells were pre-treated with blebbistatin (100μM) for 30min prior to pre-clustered EphB4-Fc treatment. Cells lost their actin stress fibres upon blebbistatin treatment and during time-lapse microscopy it was observed that slight morphological changes occured as a result of blebbistatin treatment. Cells seemed to be very flat and have thin extensions from the cell body. This phenotype has previously been referred to as a "collapsed" morphology (Wilkinson et al., 2005). The cells remained flat and the majority stayed well-spread after EphB4-Fc stimulation. A small proportion of cells showed a slight retraction phenotype, although this was not a dramatic response when compared with the effects of EphB4-Fc treatment on cells without Blebbistatin treatment. No blebbing was seen to occur (Fig. 5.1c). Quantification reveals an inhibition of retraction and blebbing to control levels, as observed after treatment with pre-clustered Fc alone (Fig 5.1d & e). Since blebbistatin is diluted in dimethyl sulfoxide (DMSO) before use, the effects of DMSO stimulation were tested to confirm that the inhibition of retraction and blebbing was a direct consequence of the inhibitor being tested. DMSO had no significant effect on the levels of retraction and blebbing seen upon EphB4-Fc stimulation (Fig. 5.1d & e).

These results indicate that cell retraction and membrane blebbing, in response to ephrin-B2 signalling, is dependent on both ROCK and actomyosin driven contractility. Phase time-lapse analysis was used to look for any dynamic effects of Y27632 and blebbistatin pre-treatment, after pre-clustered EphB4-Fc stimulation. Figures 5.2a and b show that cells are well-spread and ruffling prior to EphB4-Fc treatment. After stimulation with pre-clustered EphB4-Fc, cells pre-treated with Y27632 remained flat and continued to ruffle, with no retraction even after 45min stimulation (Fig. 5.2a; Supplementary material: Movie 5.1). Cells pre-treated with blebbistatin, prior to pre-clustered EphB4-Fc stimulation, remained well-spread, exhibiting no signs of retraction, even after 45min EphB4-Fc stimulation (Fig. 5.2b; Supplementary Material: Movie 5.2). These data can be compared favourably to the control pre-clustered Fc stimulation of HUAECs, which did not trigger retraction or blebbing (see Fig. 4.5; Supplementary material: Movie 4.3).

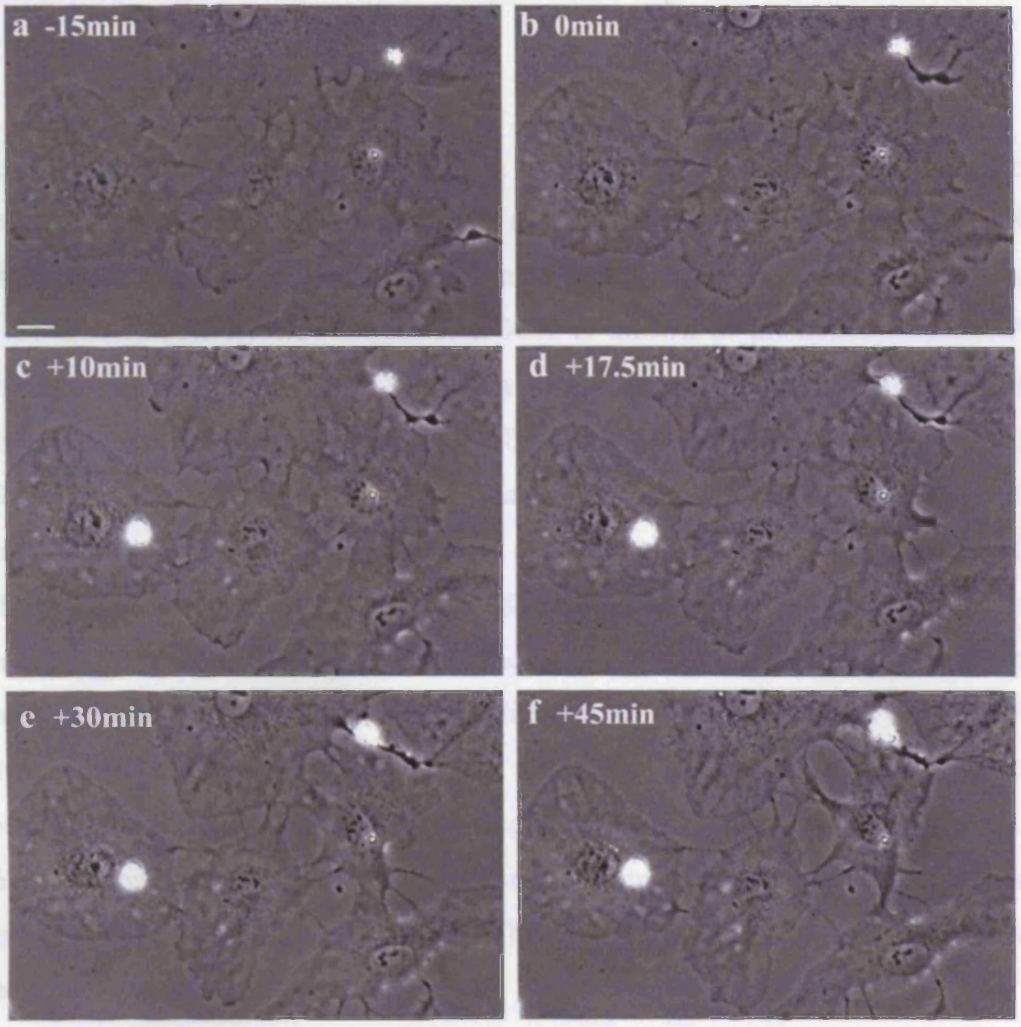
Figure 5.2 Inhibitors of ROCK and myosin II ATPase completely block retraction and blebbing triggered by ephrin-B2 activation

a) Y27632



Phase time-lapse stills showing HUAECs pre-treated with the ROCK inhibitor Y27632 (20μM) prior to pre-clustered EphB4-Fc (5μg/ml) stimulation. Unstimulated cells are well-spread and display membrane ruffling (a). Upon EphB4-Fc stimulation (b) no retraction or blebbing was observed (c-f). Even after 45min the cells remained well spread and continued to ruffle (f). Scale bar 15μm. (See Supplementary Material: Movie 5.1).

b) Blebbistatin



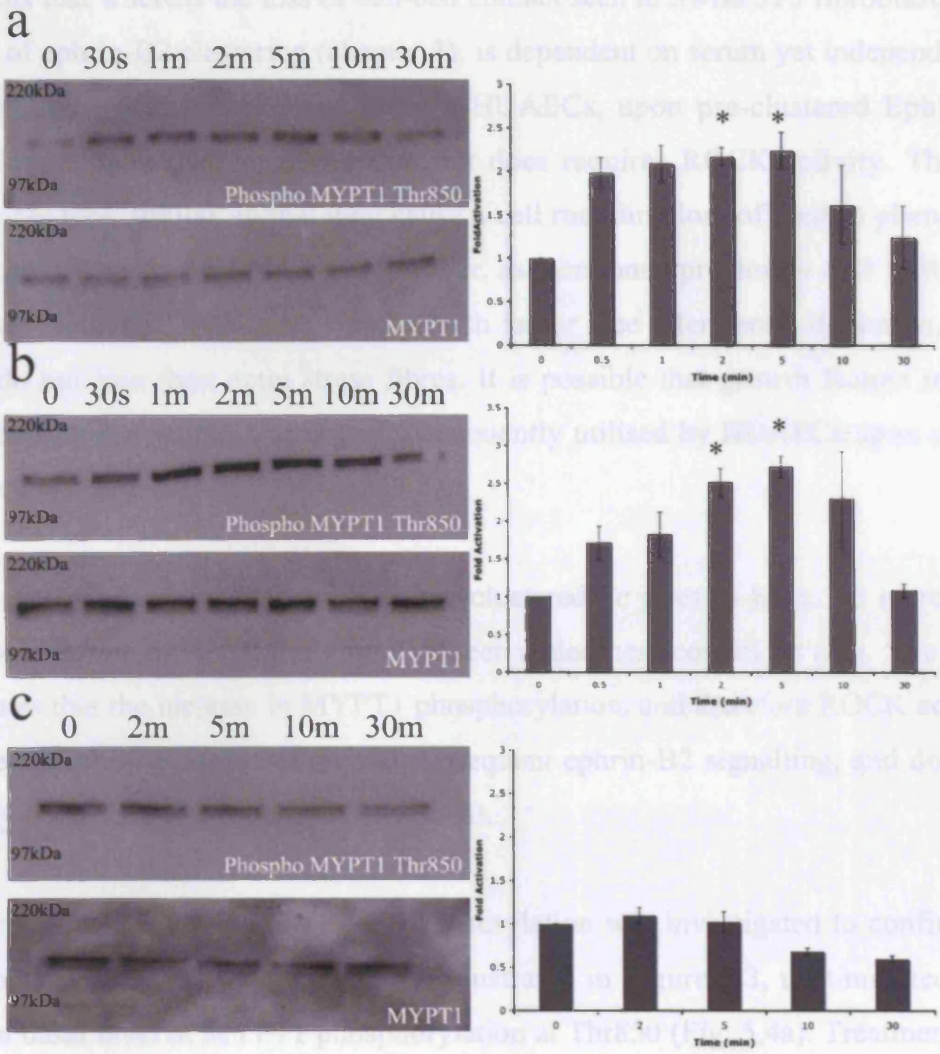
Phase time-lapse stills showing HUAECs pre-treated with Blebbistatin (100μM) prior to pre-clustered EphB4-Fc (5μg/ml) stimulation. Unstimulated cells have a well-spread, flat morphology (a). Treatment with pre-clustered EphB4-Fc (b) caused a small proportion of cells to retract slightly but did not induce any blebbing (b-f). Scale bar 15μm. (See Supplementary Material: Movie 5.2).

5.2.2 A transient increase in ROCK activity occurs in response to ephrin-B2 signalling which is blocked by the ROCK inhibitor Y27632

The data above demonstrate that ROCK is necessary for EphB4-Fc induced cell retraction and blebbing. However, it was not known whether an increase in ROCK activity occurs across the time course of retraction. In order to investigate this a read out of ROCK activity was required. ROCK is known to phosphorylate MYPT1 (Kimura et al., 1996), the 130kDa myosin phosphatase targeting subunit, which has previously been shown to be expressed by aortic endothelial cells (Hirano et al., 1999). Phosphorylation of MYPT1 inactivates myosin phosphatase allowing increased phosphorylation of myosin light chain keeping it in an active conformation and allowing actomyosin contractility (Kimura et al., 1996). Therefore, in order detect an increase in ROCK activity, phosphorylation of the targeting subunit of myosin phosphatase (MYPT1) was investigated. ROCK can phosphorylate MYPT1 at two sites: Thr696 and Thr850 (Feng et al., 1999a; Velasco et al., 2002). Western blotting was carried out at stages throughout the time course and the levels of MYPT1 phosphorylation investigated using commercially available antibodies to phosphorylated MYPT1 at Thr696 and Thr850. An antibody to total MYPT1 was used to determine correct protein loading.

Under normal culture conditions a transient increase in MYPT1 phosphorylation at Thr850 can be seen in response to EphB4-Fc stimulation (ANOVA $P = 0.0151$; Fig. 5.3a). Phosphorylation increased 30sec after stimulation, peaking at 2-5min stimulation with a >2-fold increase ($P < 0.05$). Phosphorylation then declined towards control levels by 30min. Therefore, an increase in ROCK activity appears to occur prior to retraction, with levels decreasing back to basal to allow the cells to recover. This pattern is identical under serum-starved conditions (ANOVA $P = 0.0049$; Fig. 5.3b). Once again phosphorylation peaked after 2-5min stimulation, with a >2.5-fold increase ($P < 0.05$), and declined to control levels by 30min.

Figure 5.3 Activation of ephrin-B2 triggers a transient increase in phosphorylated MYPT1 at Thr850



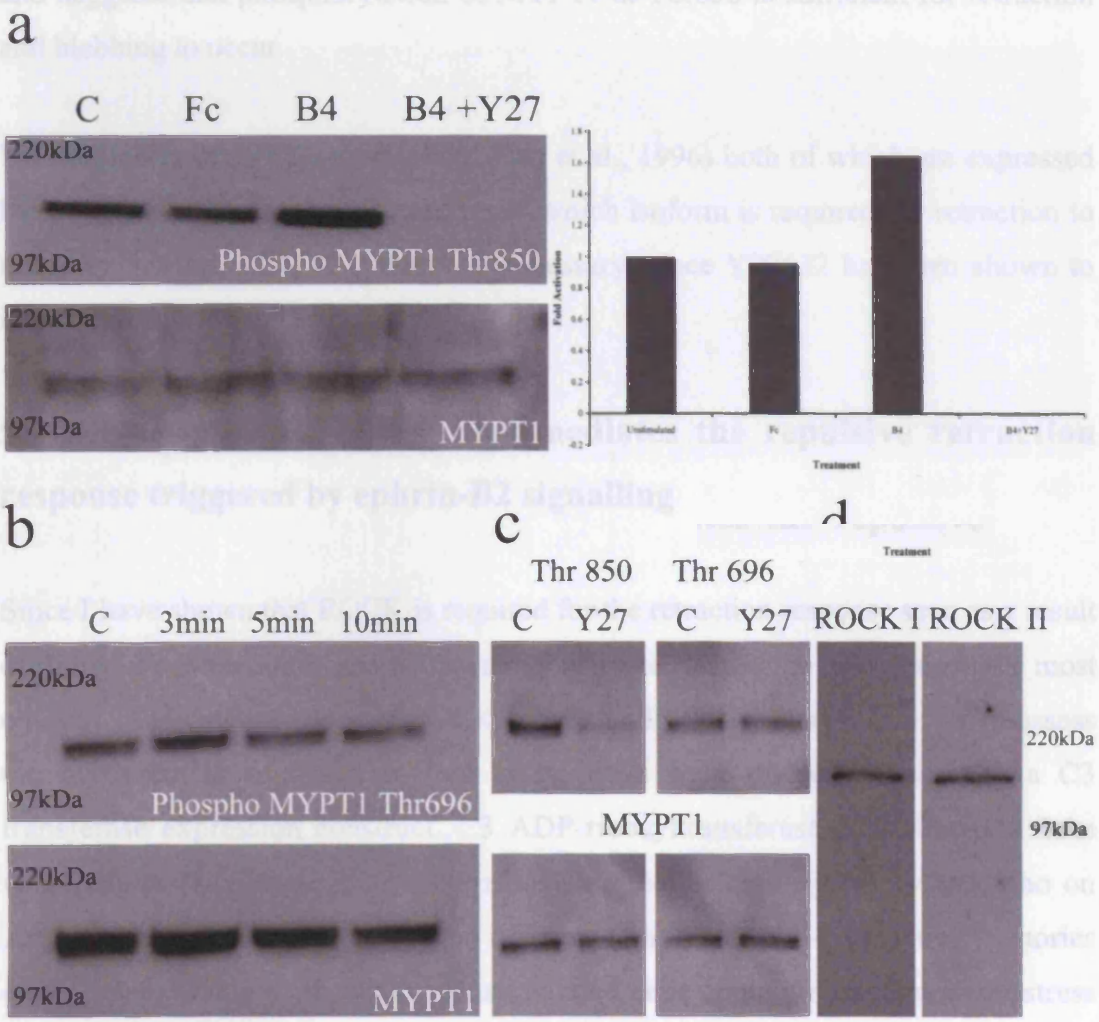
HUAECs stimulated with pre-clustered EphB4-Fc (5μg/ml) as indicated, were lysed and analysed by western blot using antiMYPT1 (115kDa) and anti-phosphoMYPT1 Thr850 (130kDa) antibodies. Equal protein loading was obtained throughout, as demonstrated by total MYPT1 levels (a-c). Under normal culture conditions a 2.1-fold increase in activation was seen after 5min EphB4-Fc stimulation ($P < 0.05$; a). Under serum-starved conditions, a >2.5-fold increase in activation ($P < 0.05$; b) was observed. The increase was transient with levels reduced to basal by 30min (a & b). As a control HUAECs were stimulated with Fc alone. No increase in phosphorylated MYPT1 at Thr850 was seen under these conditions (c). Asterisks denote statistical significance compared with unstimulated cells.

As shown in chapter 4, cells were still able to undergo retraction in the absence of serum and growth factors (see Fig. 4.9). This finding would corroborate that data, since phosphorylation of MYPT1 at Thr850 occurs with or without serum. This suggests that whereas the loss of cell-cell contact seen in Swiss 3T3 fibroblasts, as a result of ephrin-B2 clustering (chapter 3), is dependent on serum yet independent of ROCK, the retraction response seen in HUAECs, upon pre-clustered EphB4-Fc stimulation, is not serum dependent, but does requires ROCK activity. The two responses look similar, in that they cause a cell rounding/loss of contact phenotype, but occur via different pathways. However, as mentioned previously (4.3.1) it is not known whether HUAECs are truly growth factor free after serum-depletion, since cells do not lose their actin stress fibres. It is possible that growth factors may be adsorbed by the gelatin matrix and subsequently utilised by HUAECs upon serum-starvation.

As a control, cells were treated with pre-clustered Fc prior to lysis. No increase in phosphorylation of MYPT1 at Thr850 is seen under these conditions (Fig. 5.3c). This indicates that the increase in MYPT1 phosphorylation, and therefore ROCK activity, is due to EphB4-Fc stimulation and subsequent ephrin-B2 signalling, and does not simply occur as a consequence of treatment.

The effect of Y27632 on MYPT1 phosphorylation was investigated to confirm the link between MYPT1 and ROCK. As illustrated in Figure 5.3, unstimulated cells have a basal level of MYPT1 phosphorylation at Thr850 (Fig. 5.4a). Treatment with pre-clustered Fc did not affect the level of phosphorylation (Fig. 5.4a), but treatment with pre-clustered EphB4-Fc resulted in an increase in phosphorylation at Thr850 (Fig. 5.4a). However, upon pre-treatment with Y27632 (20μM) for 30min, followed by pre-clustered EphB4-Fc stimulation, phosphorylation of MYPT1 at Thr850 was completely diminished to levels below those seen in unstimulated cells (Fig. 5.4a). Interestingly, Y27632 was only found to block phosphorylation at Thr850 and not Thr696 (Fig. 5.4c) indicating that something else is phosphorylating ROCK at this site. It is known that MYPT1 can be phosphorylated by kinases other than ROCK, such as MRCK (myotonic dystrophy kinase-related Cdc42-binding kinase), a Cdc42 effector kinase (Tan et al., 2001). Supporting this lack of inhibition it was found that pre-clustered EphB4-Fc stimulation did not induce an increase in MYPT1

Figure 5.4 The ROCK inhibitor Y27632 blocks phosphorylation of MYPT1 at Thr850 but not at Thr696



HUAECs stimulated as indicated, were lysed and protein levels analysed by western blot using antibodies to either: phosphoMYPT1 Thr850 (130kDa), phosphoMYPT1 Thr696 (130kDa), total MYPT1 (115kDa), ROCK I (160kDa), or ROCK II (180kDa). No increase in phosphoMYPT1 at Thr850 above control levels was seen upon treatment with pre-clustered Fc (a). Treatment with pre-clustered EphB4-Fc (5μg/ml) caused an increase in phosphoMYPT1 at Thr850 (a). Phosphorylation at Thr850 was inhibited if cells were pre-treated with the ROCK inhibitor Y27632 (20μM; a). There was no increase in MYPT1 phosphorylation at Thr696 upon stimulation with pre-clustered EphB4-Fc (b) and Y27632 did not block phosphorylation at this site (c). HUAECs express both ROCK I and ROCK II (d).

phosphorylation at Thr696 (Fig. 5.4b). These results indicate that phosphorylation of MYPT1 at Thr696 is not required for cell retraction induced by ephrin-B2 signalling and suggests that phosphorylation of MYPT1 at Thr850 is sufficient for retraction and blebbing to occur.

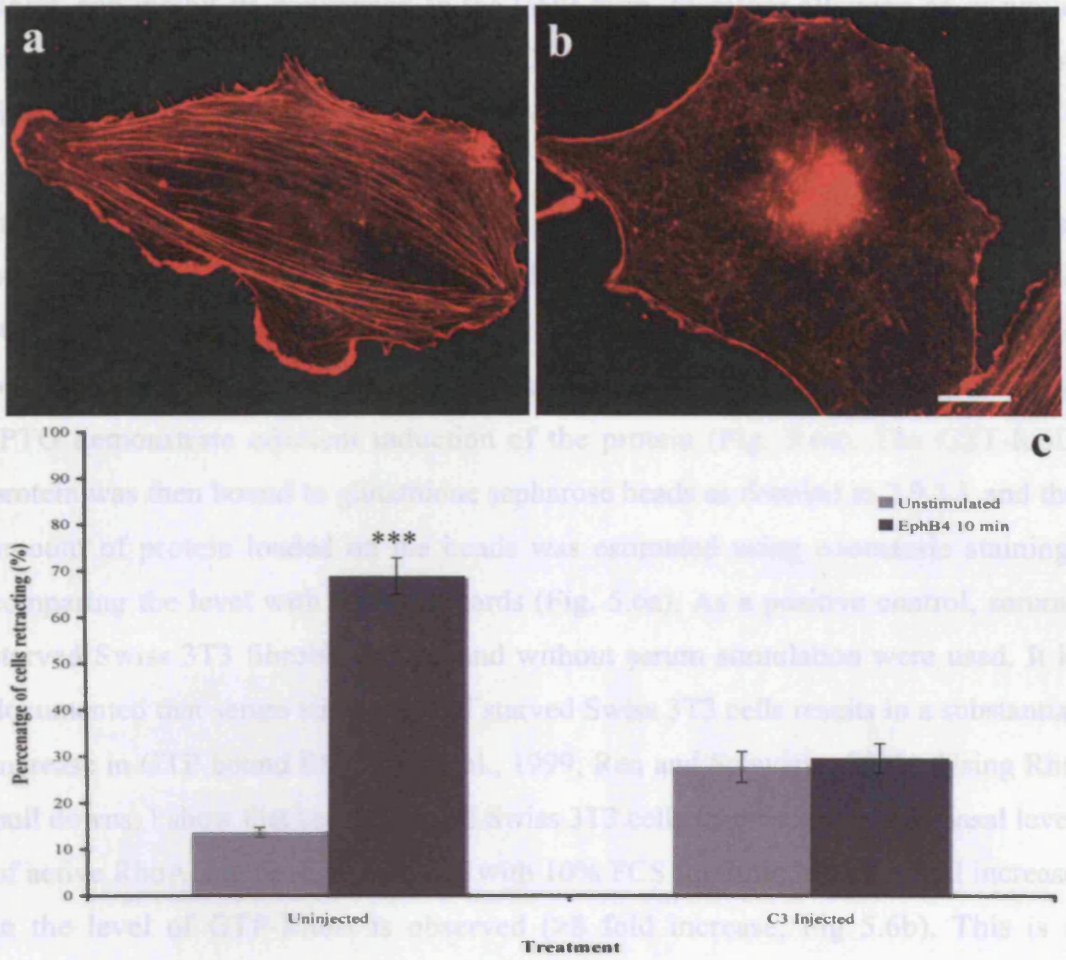
Two isoforms of ROCK exist (Nakagawa et al., 1996) both of which are expressed by HUAECs (Fig. 5.4d). It is not known which isoform is required for retraction to occur in this instance, or if both are necessary, since Y27632 has been shown to inhibit both isoforms (Ishizaki et al., 2000).

5.2.3 The small GTPase RhoA mediates the repulsive retraction response triggered by ephrin-B2 signalling

Since I have shown that ROCK is required for the retraction response seen as a result of EphB4-Fc stimulation, and is transiently activated across the time course, the most obvious candidate upstream of ROCK is the small GTPase RhoA. In order to assess the involvement of RhoA in this assay, cells were microinjected with a C3 transferase expression construct. C3 ADP-ribosyltransferase (C3), derived from *Clostridium botulinum,* is an ADP ribosylase that selectively ribosylates Rho on Asparagine and renders all three Rho isoforms (RhoA, -B and –C) inactive (Aktories et al., 1988; Wilde et al., 2000). Unstimulated cells contain numerous actin stress fibres (Fig. 5.5a). Upon injection of the C3 transferase expression construct, cells remain well-spread but lose all their actin stress fibres (Fig. 5.5b). It was found that cells injected with C3 transferase prior to pre-clustered EphB4-Fc stimulation do not undergo retraction above control levels (Fig. 5.5c). This indicates a requirement for Rho in the retraction process. Injection of this construct without EphB4-Fc stimulation did cause a slight increase in retraction (although not statistically significant) to occur compared to unstimulated cells without C3 expression. However, the levels remain constant upon EphB4 stimulation (Fig. 5.5c).

In order to look at cellular levels of active RhoA across the time course, and determine if RhoA is activated in response to pre-clustered EphB4-Fc stimulation, a technique known as a Rho pull down was carried out. This is an affinity precipitation

Figure 5.5 Inhibition of Rho via injection of C3 transferase inhibits retraction and blebbing



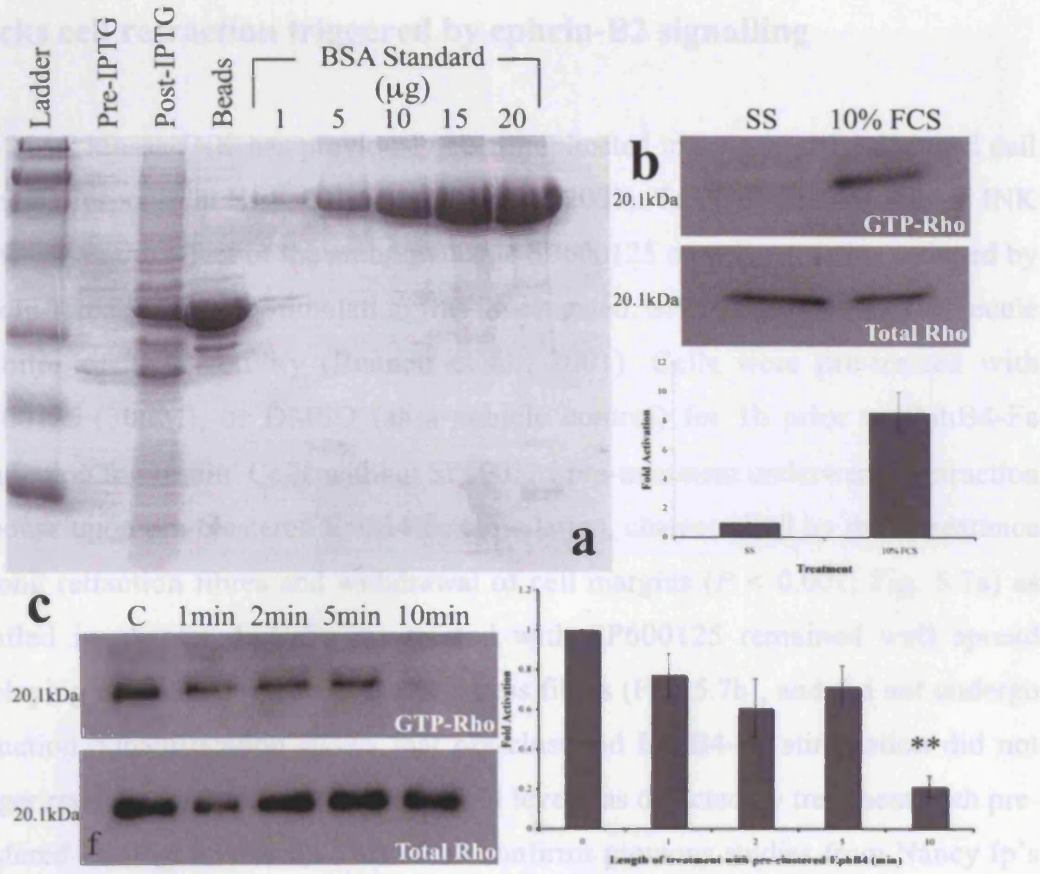
Cells were stained for F-actin using TRITC-phalloidin (red). Unstimulated HUAECs are flat well-spread cells with numerous actin stress fibres and membrane ruffles (a). Pre-clustered EphB4-Fc stimulation results in cell retraction ($P < 0.001$; c). Upon injection of C3 transferase (20μg/ml) a small proportion of cells retracted but the majority remained spread and lost their actin stress fibres (b). Cells injected with C3 transferase and subsequently stimulated with pre-clustered EphB4-Fc (5μg/ml) did not undergo retraction or blebbing at levels above those seen for unstimulated cells (c). Asterisks denote statistical significance with respect to unstimulated cells. Scale bar 15μm.

assay for endogenous GTP-loaded (active) Rho. Since Rho effectors only interact with GTP-bound Rho, the Rhotekin Rho binding domain (RBD) was used to pull down endogenous GTP-RhoA from HUAEC lysates. This domain should bind active RhoA and inhibit its conversion to the GDP form, therefore allowing an accurate measure of GTP-RhoA loading. HUAECs were stimulated for various lengths of time throughout the time course and the levels of GTP-Rho assessed.

In order to carry out this assay, I prepared glutathione sepharose beads with a fusion of the Rhotekin Rho binding domain (RBD) coupled to glutathione S-transferase (GST-RBD) bound to them. Bacteria were inoculated with GST-RBD and expression of the protein was induced with IPTG. Samples taken before and after addition of IPTG demonstrate efficient induction of the protein (Fig. 5.6a). The GST-RBD protein was then bound to glutathione sepharose beads as detailed in 2.9.3.1 and the amount of protein loaded on the beads was estimated using coomassie staining, comparing the level with BSA standards (Fig. 5.6a). As a positive control, serum-starved Swiss 3T3 fibroblasts with and without serum stimulation were used. It is documented that serum stimulation of starved Swiss 3T3 cells results in a substantial increase in GTP-bound Rho (Ren et al., 1999; Ren and Schwartz, 2000). Using Rho pull downs, I show that serum-starved Swiss 3T3 cells express a very low basal level of active RhoA, but upon stimulation with 10% FCS for 3min, a substantial increase in the level of GTP-RhoA is observed (>8 fold increase; Fig 5.6b). This is a reproducible increase, indicating the reliability of the assay.

However, throughout the time course of cell retraction, I did not observe an increase in the levels of active RhoA (Fig. 5.6c). There are a number of possible explanations for this. One possibility is that RhoA is not being activated despite mediating the response. Another is that there could be a small increase in GTP-RhoA, perhaps only a local activation, but the levels are too small to be detected here. Finally, activation may be extremely fast and I have missed it. Interestingly, at 10min I observe a dramatic reduction in GTP-bound RhoA ($P < 0.01$; Fig. 5.6c). This is reproducible and appears to coincide with the onset of recovery and loss of actin stress fibres seen in re-spreading cells. This may suggest that RhoA activation is switched off in order to allow the cells to recover.

Figure 5.6 Levels of active Rho decrease upon EphB4-Fc stimulation preceding a loss of stress fibres and re-spreading



A coomassie stained gel to show IPTG induction of the Rhotekin Rho binding domain (RBD) and the Rhotekin RBD bound to glutathione sepharose beads (a). RhoA was detected using an anti-RhoA (24kDa) antibody. As a positive control for the Rho pull down assay, serum-starved (SS) Swiss 3T3 fibroblasts were used, which contain very little if any GTP-bound Rho (a). Upon stimulation with 10% FCS for 3min a >8-fold induction of GTP-bound Rho occurred (a). The levels of total Rho were equal in the two samples (a). Upon stimulation of HUAEC lysates with pre-clustered EphB4-Fc (5μg/ml) no increase in Rho-GTP was observed. Interestingly, the levels of active Rho declined (c). The effect was most dramatic at 10min resulting in a substantial decrease in GTP-bound Rho (c). This can be quantified,

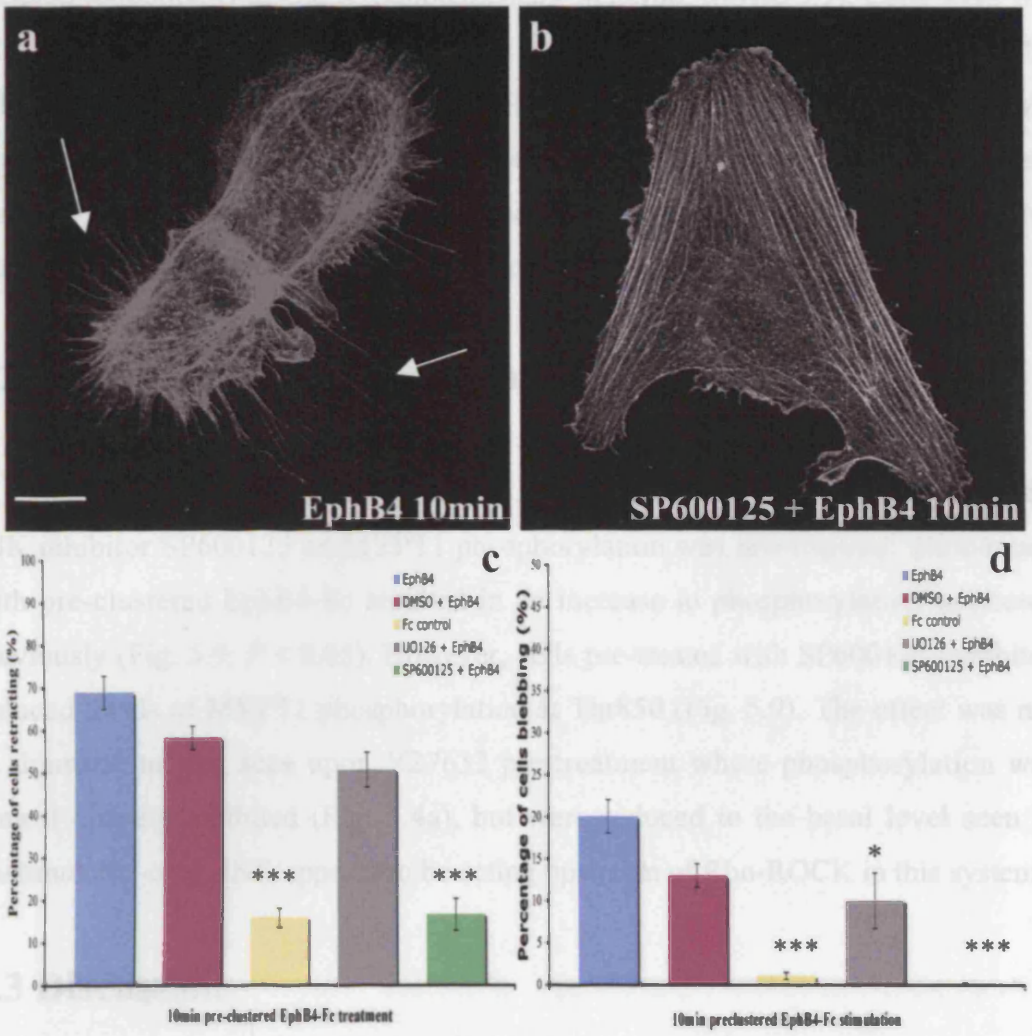
illustrating a 4-fold decrease with respect to control ($P < 0.01$; g). Asterisks denote statistical difference with respect to unstimulated cells.

5.2.4 The c-Jun amino terminal kinase (JNK) inhibitor SP600125 blocks cell retraction triggered by ephrin-B2 signalling

The MAP kinase JNK has previously been implicated in an ephrin-B1-induced cell rounding response in HEK 293 cells (Xu et al., 2003). To determine the role of JNK in this assay the effect of the anthrapyrazole SP600125 on cell retraction induced by pre-clustered EphB4-Fc stimulation was investigated. SP600125 is a small molecule inhibitor of JNK activity (Bennett et al., 2001). Cells were pre-treated with SP600125 (30μM), or DMSO (as a vehicle control) for 1h prior to EphB4-Fc stimulation for 10min. Cells without SP600125 pre-treatment underwent a retraction response upon pre-clustered EphB4-Fc stimulation, characterised by the appearance of long retraction fibres and withdrawal of cell margins ($P < 0.001$; Fig. 5.7a) as detailed in chapter 4. Cells pre-treated with SP600125 remained well spread displaying membrane ruffles and actin stress fibres (Fig. 5.7b), and did not undergo retraction. Quantification shows that pre-clustered EphB4-Fc stimulation did not trigger retraction or blebbing above control levels, as depicted by treatment with pre-clustered Fc (Fig. 5.7c & d). This result confirms previous studies from Nancy Ip's laboratory demonstrating that JNK is required for cell rounding in response to ephrin-B activation (Xu et al., 2003).

In order to test whether this inhibition is specific to the MAP kinase JNK, another MAP kinase inhibitor was also used. UO126 is a selective inhibitor of the mitogen activated protein kinase kinases MEK-1 and MEK-2 (Favata et al., 1998). Pre-treatment with UO126 (20μM) for 30min prior to EphB4-Fc stimulation did not result in an inhibition of retraction and only slightly affected the levels of blebbing ($P < 0.05$; Fig. 5.7c & d). This suggests that the retraction response observed may be specific to the JNK pathway, although inhibitors to every MAP kinase were not tested. As a control, cells were pre-treated with DMSO alone prior to stimulation, since both inhibitors were diluted in DMSO and it was important to ascertain that the

Figure 5.7 Membrane retraction and blebbing triggered by EphB4 treatment are blocked by the c-jun amino terminal kinase (JNK) inhibitor SP600125



Cells were stained for F-actin using TRITC-phalloidin. HUAECs stimulated with pre-clustered EphB4-Fc (5μg/ml) for 10min retract their cell margins leaving behind long retraction fibres (arrows in a). Pre-treatment of cells with the JNK inhibitor SP600125 (30μM) blocked the retraction response. Cells remained flat and contained actin stress fibres (b) and cell retraction/blebbing was reduced to control levels, seen with pre-clustered Fc treatment alone (c & d). The MAP kinase inhibitor UO126 did not block retraction and did not inhibit blebbing to the same extent (c & d). EphB4-Fc stimulation was not effected by the presence of DMSO (c & d). Asterisks denote statistical difference with respect to EphB4 stimulation. Scale bar 15μm.

carrier itself was not exerting any inhibitory effects on the process. No significant effects on the levels of retraction and blebbing were seen (Fig. 5.7c & d).

Phase time-lapse analysis confirms that cells pre-treated with SP600125 do not undergo retraction (Fig. 5.8 & Supplementary Material: Movie 5.3). Cells were flat with membrane ruffles prior to treatment. Upon stimulation the morphology of the cells was not altered, no differences in cell behaviour were noted even after 45min stimulation (Fig. 5.8 & Supplementary Material: Movie 5.3). This lack of retraction response can be compared favourably to that seen when cells are stimulated with pre-clustered Fc as a control (Fig. 4.5; Supplementary Material: Movie 4.3).

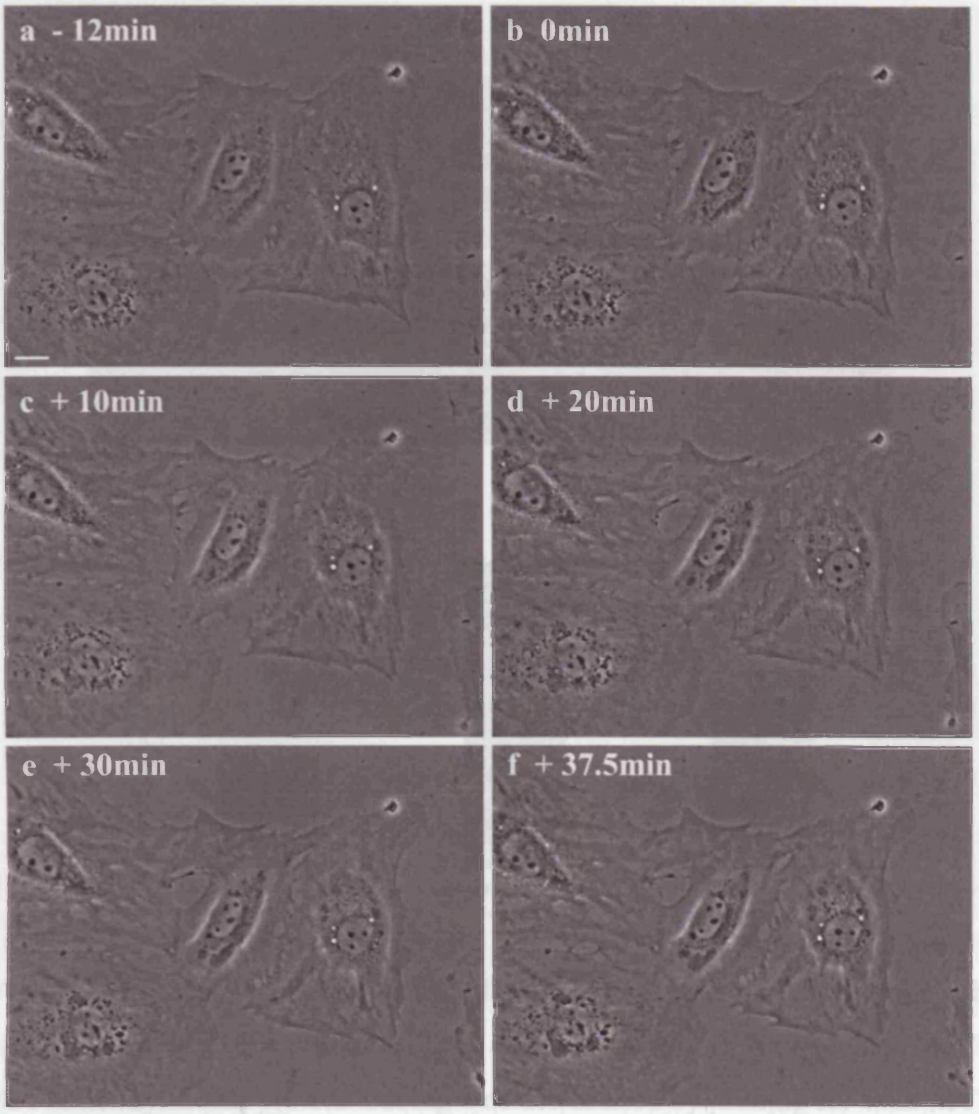
5.2.5 JNK is acting upstream of Rho

In an attempt to determine where JNK lies in the signalling pathway the effect of the JNK inhibitor SP600125 on MYPT1 phosphorylation was investigated. Stimulation with pre-clustered EphB4-Fc resulted in an increase in phosphorylation as shown previously (Fig. 5.9; $P < 0.05$). However, cells pre-treated with SP600125 exhibited reduced levels of MYPT1 phosphorylation at Thr850 (Fig. 5.9). The effect was not as dramatic as that seen upon Y27632 pre-treatment where phosphorylation was almost entirely inhibited (Fig. 5.4a), but were reduced to the basal level seen in unstimulated cells. JNK appears to be acting upstream of Rho-ROCK in this system.

5.3 Discussion

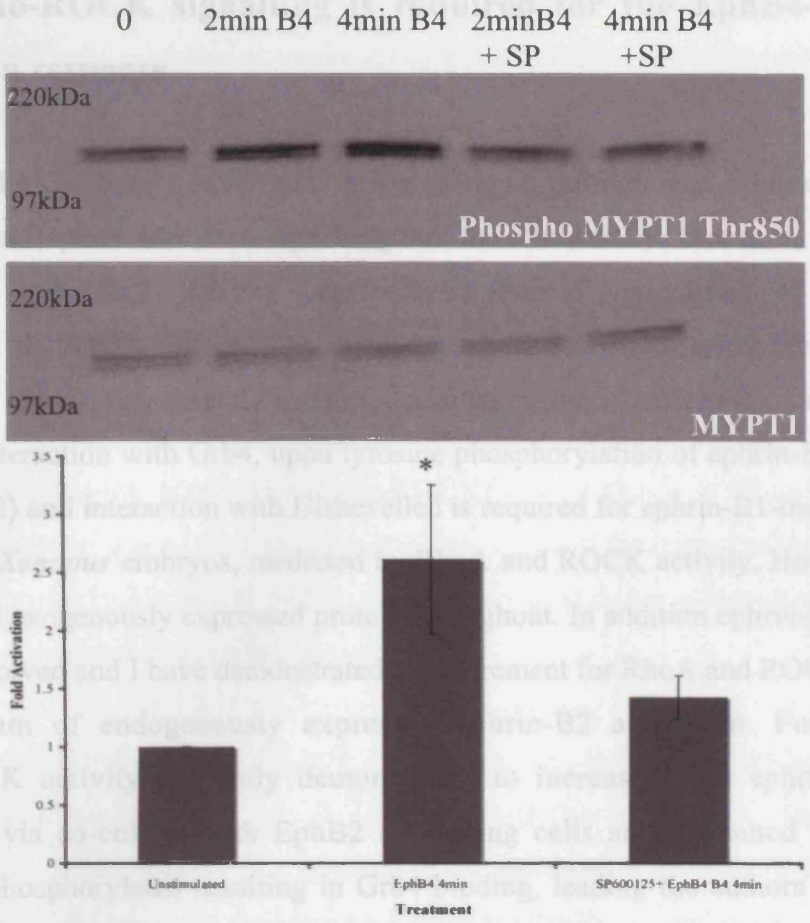
In this chapter, I have described a requirement for Rho, ROCK and JNK activity, together with acto-myosin contractility, for the retraction seen upon ephrin-B2 activation in HUAECs. Inhibiton of ROCK and myosin II ATPase prevents retraction and blebbing in response to pre-clustered EphB4-Fc stimulation. A transient increase in MYPT1 phosphorylation at Thr850 is seen across the time course of retraction, which can be blocked using the ROCK inhibitor Y27632. The small GTPase Rho is required for retraction to occur and there is a substantial down-regulation of active Rho coincident with re-spreading, suggesting active Rho is

Figure 5.8 The JNK inhibitor SP600125 blocks EphB4 triggered cell retraction



Phase time-lapse stills of HUAECs pre-treated with SP600125 (30μM) and stimulated with pre-clustered EphB4-Fc (5μg/ml). Unstimulated cells are flat, well-spread and display membrane ruffling (a). Stimulation with EphB4-Fc (b) did not induce membrane retraction (c-f). Prolonged stimulation with pre-clustered EphB4-Fc for >35min did not trigger a retraction response (f). Scale bar 15μm. (See Supplementary Material: Movie 5.3).

Figure 5.9 The JNK inhibitor SP600125 inhibits EphB4 triggered phosphorylation of MYPT1 at Thr850



HUAECs were stimulated as indicated, lysed and analysed by western blot. Phosphorylated MYPT1 (130kDa) was detected using an anti-phosphoMYPT1 Thr850 antibody. Equal protein loading was determined by probing for total MYPT1 (115kDa). Stimulation with pre-clustered EphB4-Fc (5μg/ml) caused an increase in phosphoMYPT1 at Thr850 ($P < 0.05$). Pre-treatment with the JNK inhibitor SP600125 (30μM) inhibited the phosphorylation seen in response to EphB4-Fc stimulation. Asterisks denote statistical difference with respect to unstimulated cells.

switched off to allow cells to recover. JNK appears to be acting upstream of Rho-ROCK in this pathway.

5.3.1 Rho-ROCK signalling is required for the EphB4-induced retraction response

Although Eph receptors have been shown to signal through Rho GTPases, little is known about ephrin mediated signalling and the Rho family. However, during my PhD, Rho and ROCK activity have been implicated downstream of ephrin-B1 (Tanaka et al., 2003). *Xenopus* Dishevelled has been shown to form a complex with ephrin-B1 via binding directly to the C-terminal region of ephrin-B1, or indirectly through interaction with Grb4, upon tyrosine phosphorylation of ephrin-B1 (Tanaka et al., 2003) and interaction with Dishevelled is required for ephrin-B1-mediated cell sorting in *Xenopus* embryos, mediated by RhoA and ROCK activity. However, this study used exogenously expressed protein throughout. In addition ephrin-B1 was the ligand involved and I have demonstrated a requirement for RhoA and ROCK activity downstream of endogenously expressed ephrin-B2 activation. Furthermore, Rho/ROCK activity was only demonstrated to increase when ephrin-B1 was activated via co-culture with EphB2 expressing cells and presumed to become tyrosine phosphorylated resulting in Grb4 binding, leading the authors to suggest Grb4 is involved. However, I have demonstrated cell rounding independently of Grb4 as a consequence of ephrin-B2 signalling. Very recently a second paper has implicated RhoA downstream of ephrin-B1 (Lee et al., 2006). Ephrin-B1 was shown to act through the PCP pathway via Dishevelled during *Xenopus* development to control the migration of cells into the eye field. Both studies were carried out in *Xenopus*. It remains to be seen whether Dishevelled mediates Rho/ROCK activation downstream of ephrin-B2 in mammalian cells. Daam-1, a formin homology protein which forms a complex with Dvl (the mammalian homologue of Dishevelled) and Rho in mammalian cells could be a potential candidate (Habas et al., 2001).

I have demonstrated a requirement for Rho to enable HUAECs to retract in response to EphB4 stimulation. However, using the Rho pull down technique I was unable to demonstrate an increase in active RhoA. There are a number of possible reasons for

this. Firstly, activation may be very rapid, occurring prior to the 30sec time point in my assay, and I have therefore missed it. Secondly cells grown in serum have high basal levels of GTP-Rho due to activation by serum lipids such as LPA and so if the increase in active RhoA is small, possibly via a local activation at specific sites, it may be hard to detect it in this assay. Although HUAECs are serum-starved for the Rho pull down assay, prior to EphB4-Fc treatment and lysis, they do not lose their actin stress fibres. The gelatin matrix may bind serum factors that can be utilised upon serum-starvation, rendering it less effective. This is implied by the substantial level of Rho-GTP present in unstimulated HUAECs compared to that in serum-starved Swiss 3T3 fibroblasts (Fig. 5.6b & e). Thirdly, GTP-RhoA may be necessary for the ephrinB2-triggered retraction, but RhoA is not activated by ephrin-B2. If this was the case, ROCK would be activated independently of Rho. Some lipids, in particular arachidonic acid, can activate ROCKs independently of Rho (Feng et al., 1999b). However, due to the finding that Rho is required for retraction to take place, as demonstrated by the failure of C3 injected cells to undergo retraction in response to EphB4 stimulation, this seems unlikely.

Upon EphB4 stimulation of HUAECs, I have shown an increase in ROCK activity, a downstream effector of Rho, which correlates with the onset of retraction. ROCK activity is transient, reducing to basal levels at the time when cells re-spread their lamellae. ROCK activity is therefore necessary for EphB4 triggered retraction and is down regulated upon recovery. Despite the lack of a demonstrable increase in the levels of active RhoA at the onset of retraction, a striking finding is that active RhoA is switched off at later times coincident with the loss of actin-stress fibres and re-spreading. It is possibly that downregulation of RhoA could occur in order to allow recovery to take place. This could occur via recruitment of a RhoGAP, which would promote the hydrolysis of GTP to GDP, inactivating the protein. However, recovery could also be the result of other factors, such as internalisation of the ephrin ligand or Grb4, since we have recently found a requirement for Grb4 in order for re-spreading to occur (Nobes lab data).

Evidence already exists for a role of RhoA downstream of EphA and, to a lesser extent, EphB receptors. For example, ephrin-A5 stimulation of retinal ganglion cells induces Rho-ROCK dependent growth cone collapse (Wahl et al., 2000). In addition

stimulation of EphA3 expressing 293T cells with ephrin-A5 has been shown to induce rounding, blebbing and de-adhesion, which is mediated by RhoA signalling (Lawrenson et al., 2002). Parallels can be seen between these responses, which require Rho-ROCK mediated actin-myosin contractility to generate cell/axon retraction, and the retraction response I describe after ephrin-B2 stimulation in HUAECs. One molecule that links RhoA to EphA receptors in neurons is Ephexin, a guanine nucleotide exchange factor that activates RhoA. Ephexin is known to bind to EphA4 for example, and becomes phosphorylated upon ephrin-A stimulation leading to RhoA activation and mediation of growth cone dynamics (Sahin et al., 2005; Shamah et al., 2001). In addition, Vms-Rho-GEF is another exchange factor that binds to EphA4 and mediates RhoA activation in rat vascular smooth muscle cells (Ogita et al., 2003). It is possible that a Rho-GEF is recruited upon ephrin-B2 activation that could link ephrin-B2 to RhoA.

EphB receptors have also been linked to Rho. *Xenopus* Dishevelled associates with activated EphB1 and EphB2 and stimulation of EphB2 leads to Rho-ROCK activation which is dependent on Dishevelled (Tanaka et al., 2003). In addition, the Ras GTPase activating protein p120RasGAP can bind to activated EphB receptors (Holland et al., 1997) and has also been shown to associate with p190RhoGAP (Settleman et al., 1992) which, for example, is known to deactivate RhoA downstream from integrins (Arthur and Burridge, 2001). I observe down-regulation of RhoA upon re-spreading, after EphB4-induced retraction in HUAECs, as a consequence of ephrin-B2 signalling. This could be regulated by a RhoGAP such p190RhoGAP, known to inhibit Rho-induced stress fibre formation (Ridley et al., 1993), which has been shown to bind to PTP-BL (Saras et al., 1997) a phosphotyrosine phosphatase which is known to associate with activated ephrin-Bs (Palmer et al., 2002). Alternatively a PDZ-RhoGEF may be recruited to the PDZ binding motif of ephrin-B2, which would occur independently of tyrosine phosphorylation. Since I have shown that retraction of HUAECs in response to ephrin-B2 signalling is independent of Grb4, this suggests that retraction is also independent of tyrosine phosphorylation, since Grb4 is known to bind to ephrin-B1 in a phosphotyrosine-dependent manner (Cowan and Henkemeyer, 2001). There is currently no evidence for the recruitment of a PDZ-RhoGEF to Eph receptors or ephrins. However, plexin-Bs, transmembrane proteins which, similarly to ephrin-Bs,

lack any known catalytic activity of their own, have been shown to mediate RhoA activation via PDZ-Rho-GEFs after stimulation with their ligands, the semaphorins (Driessens et al., 2002; Perrot et al., 2002; Swiercz et al., 2002).

5.3.2 JNK is required upstream of Rho-ROCK for retraction triggered by ephrin-B2 signalling

JNK has previously been implicated in cell rounding as a consequence of ephrin-B1 activation (Xu et al., 2003). In this instance cell rounding occurs independently of tyrosine phosphorylation and Grb4 but requires JNK activity (Xu et al., 2003). My findings corroborate those of Xu and colleagues. I have demonstrated the requirement of JNK activity for retraction and blebbing as a consequence of ephrin-B2 signalling, and the retraction induced is independent of Grb4 (see chapter 4). In addition, I have demonstrated that JNK activity is needed for ROCK to be activated in response to EphB4 stimulation, indicating that JNK is acting upstream of Rho/ROCK.

Links between JNK and Rho have been previously reported. For example, the c-jun amino-terminal kinase interacting protein-1 (JIP-1) has been found to interact with p190RhoGEF in neurons (Meyer et al., 1999). JIP-1 is a known JNK substrate (Dickens et al., 1997), and has been reported to be a scaffolding protein for the formation of a JNK activating complex (Dong et al., 1998). Activation of RhoA or over-expression of Rho-GEF in neuronal cells causes neurite retraction and cell rounding (Gebbink et al., 1997), and this together with the localisation of JIP-1 to the tips of neurites in NIE-115 and PC12 cells, suggested that JIP-1 may function as a scaffold protein to localise Rho-GEF into a position to interact with RhoA, and regulate neurite retraction (Meyer et al., 1999). It is possible that a protein such as JIP-1 may link a Rho-GEF to RhoA downstream of ephrin-B2 in the retraction response seen to ephrin-B2 aignalling in HUAECs.

Previous reports of JNK activation downstream from Eph receptors and ephrins have been described. As already mentioned, Xu and colleagues have reported JNK activation to be a crucial component of the cell rounding seen in response to ephrin-

B1 activation in 293T cells (Xu et al., 2003). Also, JNK activation has been shown to occur downstream from ephrin-B1 which is required for integrin-mediated cell attachment (Huynh-Do et al., 2002). In addition, EphB1 has been linked to JNK activation via recruitment of the SH2 domain adaptor protein Nck (Stein et al., 1998a) and more recently, Ste20 kinase Nck interacting protein (NIK) has been shown to be activated by EphB1 and EphB2 and its activation is required for EphB1-stimulated JNK activation, presumably via Nck, and integrin mediated cell attachment to fibrinogen (Becker et al., 2000).

5.3.4 Conclusions

I have reported cell rounding of HUAECs in response to ephrin-B2 signalling that requires JNK activation and Rho-ROCK signalling. Activation of JNK and subsequent ROCK activation, presumably via Rho, leads to a cell contraction event that is dependent on actomyosin contractility and occurs independently of the adaptor protein Grb4. This response is transient. Down-regulation of Rho activity is coincident with a loss of actin stress fibres and re-spreading. RhoA downregulation may be required for recovery to take place, but this would need to be tested. These data corroborate those of Xu and colleagues who show a cell rounding response to ephrin-B1 activation that requires JNK activity but is independent of Grb4 (Xu et al., 2003).

Interestingly, I have also elucidated a function of JNK upstream from Rho-ROCK signalling to cause actomyosin contractility. Rho-ROCK signalling has previously been reported to function downstream of ephrin-B1 in *Xenopus,* where Dishevelled was shown to mediate ephrin-B1 regulation of cell repulsion via the activation of both RhoA and ROCK (Tanaka et al., 2003). My data provide a pathway where JNK activation and Rho-ROCK signalling function together to provide a mechanism for cell retraction as a result of ephrin-B2 stimulation by EphB4.

Chapter 6

General Discussion

The aim of this study was to investigate ephrin-B reverse signalling to the actin cytoskeleton, and to try and understand how this impinges on cell motility and migration throughout development and in the adult. Eph/ephrin signalling has been shown to function during many developmental processes, and Eph receptors and ephrins are expressed in a large number of tissues. I have focused my study on the ligand ephrin-B2 and its activation by two Eph receptors, EphB2 and EphB4.

Stimulation of exogenous ephrin-B2 expressed in confluent, quiescent, Swiss 3T3 fibroblasts by treatment with soluble, pre-clustered EphB2-Fc or EphB4-Fc triggered clustering of ephrin-B2 into distinct membrane patches, and a loss of cell-cell contact within 10min. Maximal loss of contact occurred after 1h and the response did not appear to be transient. Loss of cell-cell contact triggered by EphB2-Fc treatment was dependent on serum factors or FGF and the adaptor protein Grb4, with Src-dependent tyrosine phosphorylation of ephrin-B2 required for a full response. Neither interaction with PDZ domain proteins, or activation of either the Rho effector ROCK, or c-jun amino terminal kinase (JNK), was needed for loss of cell-cell contact.

Interestingly, stimulation of sub-confluent, cultured endothelial cells that endogenously express ephrin-B2, with soluble, pre-clustered EphB4-Fc triggered rapid cell retraction and the appearance of long actin-rich retraction fibres. A proportion of cells also exhibited signs of membrane blebbing. The response was transient, cells recovered to re-spread lamellae, concomitant with a loss of actin stress fibres within 30min, in the continued presence of EphB4-Fc. In endothelial cells, EphB4 induced membrane retraction required the cytoplasmic domain of the ligand, actomyosin driven contraction, Rho/ROCK and JNK activity, but occurred independently of serum factors and Grb4. A dramatic down-regulation of Rho activity was observed, coincident with loss of actin stress fibres and cell recovery to a well-spread morphology.

6.1 Two distinct signalling pathways downstream of ephrin-B2

I have shown that ephrin-B2 signalling can trigger two distinct pathways. In HUAECs a cell retraction response occurs, independently of serum factors and Grb4 but dependent on JNK, Rho and ROCK activity. This corroborates the findings of Xu and colleagues, who reported a cell rounding response to activation of ephrin-B1 in transfected HEK 293 cells, that was dependent on JNK activity, yet independent of Grb4 (Xu et al., 2003). The retraction response I describe in endothelial cells after ephrin-B2 signalling is transient, followed by re-spreading of lamellae coincident with a loss of actin stress fibres. In Swiss 3T3 cells, exogenously expressing ephrin-B2, Grb4-dependent loss of cell-cell contact is triggered by ephrin-B2 clustering, which requires serum factors yet is independent of JNK and ROCK activity. Src-dependent tyrosine phosphorylation is necessary for the full response to occur. These findings corroborate those of Cowan and colleagues who reported a cell rounding phenotype, together with a loss of actin stress fibres, as a consequence of ephrin-B1 activation in BHK cells, that was dependent on Grb4 binding in a tyrosine phosphorylation dependent manner (Cowan and Henkemeyer, 2001). Interestingly, although the signalling downstream of ephrin-B2 in HUAECs corroborates work from Nancy Ips laboratory (Xu et al., 2003), the loss of actin stress fibres found upon re-spreading would corroborate the actin reorganisations described in BHK cells after ephrin-B1 activation (Cowan and Henkemeyer, 2001).

In both cell types, a repulsive response is triggered by ephrin-B2 signalling, but the retraction and loss of cell-cell contact are brought about via different signalling pathways. There are a number of possible explanations for this. Signalling downstream of ephrin-B2 may be cell type dependent. In endothelial cells, a physiologically relevant cell type, since ephrin-B2 is known to play a role in vascular development *in vivo* (Gerety and Anderson, 2002), ephrin-B2 stimulation results in a dramatic contraction event. However, expression in Swiss 3T3 fibroblasts, leads to loss of cell-cell contact after ephrin-B2 clustering. In addition, the confluency of the cells may contribute to the different responses observed. Confluent Swiss 3T3 fibroblasts not only possess cell-matrix adhesions they also have cell-cell adhesions as they are stimulated as a confluent, quiescent monolayer. Sub-confluent HUAECs

only have to sever cell-matrix adhesions in order to retract. Alternatively, the level of ephrin-B2 expression may be important. In HUAECs endogenous levels of ligand are stimulated where as in Swiss 3T3 fibroblasts ephrin-B2 was overexpressed, presumably resulting in higher expression levels, and rapid formation of higher order clustered ephrins. Finally, two different receptors were used to stimulate ephrin-B2. EphB4 stimulation may trigger an actomyosin contraction pathway, whereas EphB2-Fc triggers a Grb4, tyrosine phosphorylation dependent pathway.

6.2 Ephrin-B2 regulation of the actin cytoskeleton through the Rho family of small GTPases

There are previous reports of Eph receptor and ephrin-A activation resulting in RhoA activation (Lawrenson et al., 2002; Tanaka et al., 2003), and potential RhoGEFs mediating this have been described (Sahin et al., 2005; Shamah et al., 2001). Previous evidence that ephrin-B activation triggers activation of Rho and ROCK is limited. However during my PhD, a study in *Xenopus* reported Rho and ROCK activity downstream of exogenous ephrin-B1 activation that is mediated by Dishevelled and necessary for the sorting of EphB2 and ephrin-B1 expressing cells during an in vitro re-aggregation assay (Tanaka et al., 2003). This study suggested that the increase in Rho/ROCK activity upon co-culture of ephrin-B1 expressing cells with EphB2 expressing cells could require tyrosine phosphorylation of ephrin-B1 and Grb4 binding since although Dishevelled constitutively binds to ephrin-B1, no increase in Rho activity is seen without ephrin-B1 activation.

I have demonstrated a requirement for Rho activity and activation of ROCK downstream of endogenous ephrin-B2 signalling in HUAECs, in order for cell retraction and membrane blebbing to occur. However, cell retraction in HUAECs is Grb4 independent. A second study has very recently described a role for ephrin-B1 during *Xenopus* development to control the migration of cells into the eye field, through the PCP pathway mediated via Dishevelled. Both of these studies were carried out in *Xenopus*. A role for Dishevelled in mediating RhoA activity downstream of ephrin-Bs in mammalian cells remains to be determined. Interestingly, coincident with the onset of recovery and loss of actin stress fibres

observed in HUAECs stimulated with pre-clustered EphB4, I observe a decrease in both Rho and ROCK activity, which may be necessary for cell re-spreading to take place.

It remains to be determined whether ephrin-B2 signalling in HUAECs causes RhoA activation, or if GTP-RhoA is simply recruited to sites of ephrin-B2 activation. Similarly, the identity of a potential RhoGAP that could be responsible for the down regulation of active RhoA at the onset of recovery remains elusive. Interestingly, although Grb4 is not required for cell retraction after ephrin-B2 stimulation, it is necessary for the later re-spreading (data from Nobes lab). Therefore, Grb4 could be required for the down-regulation of active Rho and this enables cells to recover, or an alternative could be that Grb4 is needed for internalisation of the ligand, which could be essential for cell re-spreading to occur.

6.3 JNK activity upstream of Rho/ROCK

Ephrin-B1 activation has previously been shown to mediate a cell rounding response after ephrin-B1 activation in HEK 293 cells (Xu et al., 2003). Ephrin-B1 activation has also been shown to induce JNK phosphorylation and modulate integrin-mediated cell attachment to fibrinogen (Huynh-Do et al., 2002). Interestingly, the retraction response observed after ephrin-B2 stimulation in HUAECs involves a loss of adhesion. This could be mediated by integrins. Although JNK activity has been previously linked to both Eph receptors and ephrins (Becker et al., 2000; Stein et al., 1998a), a role upstream of Rho/ROCK activation as a consequence of ephrin-B activation has not been documented. I have shown that JNK inhibition after EphB4 stimulation of HUAECs blocks any increase seen in ROCK activity. It is possible that a Rho-GEF is recruited that can link JNK with Rho, such as p190RhoGEF, which has been found to interact with JIP-1 (the c-jun amino-terminal kinase interacting protein-1) in neurons (Meyer et al., 1999).

6.4 Ephrin-B2 signalling in the development of the mammalian vasculature

In vivo genetic studies have definitively demonstrated the importance of the receptor-ligand partnership of ephrin-B2 and EphB4, which has been shown to play essential roles during the development of the mammalian vasculature. Endothelial cells expressing EphB4 or ephrin-B2 come into contact during blood vessel remodelling, resulting in the formation of separate arteries and veins, presumably through repulsive cell-cell interactions (Adams et al., 1999; Gerety et al., 1999; Wang et al., 1998), and cardiovascular ephrin-B2 has been shown to be essential for correct vascular remodelling, mesenchymal expression of the ligand cannot compensate for loss of ephrin-B2 in the vasculature (Gerety and Anderson, 2002). In addition, ephrin-B2 mutant mice have recently been shown to exhibit defects in the migration of smooth muscle cells to lymphatic capillaries suggesting an *in vivo* requirement for ephrin-B2 for correct cell migration. (Foo et al., 2006). The evidence I have presented, and work from other groups has shed light on how EphB/ephrin-B interactions could regulate the cell migration and repulsive interactions needed for arterial and venous cells to reach their correct targets.

Supporting the idea that EphB4/ephrin-B2 signalling is necessary for cell-cell repulsive events to form boundaries between arteries and veins, two endothelial cell populations, one expressing ephrin-B2 and the other EphB4, segregate when mixed (Fuller et al., 2003), suggesting the maintenance of boundaries as a result of EphB4-ephrin-B signalling. However, *in vitro* experiments using ephrin-B2 expressing endothelial cells stimulated with EphB4, have led to conflicting results. Ephrin-B activation has been shown to result in both attractive and repulsive effects on cell migration and adhesion. Ephrin-B2 activation by stimulation with EphB4-Fc has been reported to increase cell migration, attachment and proliferation (Fuller et al., 2003; Hamada et al., 2003; Steinle et al., 2003). However, growing ephrin-B2 expressing cells on EphB4-expressing cells inhibits sprouting angiogenesis (Zhang et al., 2001). The reasons behind the different cellular outcomes downstream of ephrin-B2 signalling not clear. Experimental conditions have varied as well as the cell type used. Endothelial cells of different vascular beds may possess a unique complement

of Eph receptors and ephrins, which determines the resultant signalling pathways upon activation.

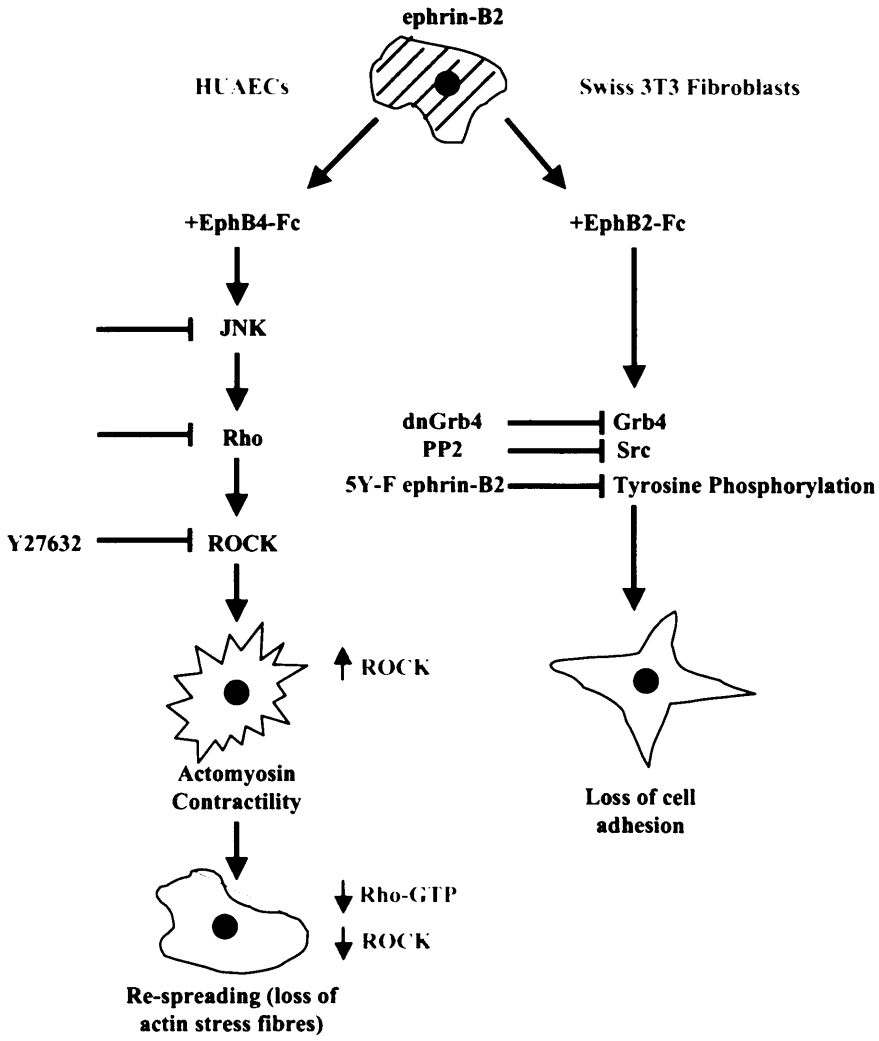
The findings of this study describe a repulsive cell retraction and loss of matrix adhesion phenotype in response to ephrin-B2 signalling, which is followed by rapid re-spreading. Cycles of retraction and protrusion of endothelial cells expressing ephrin-B2 may be necessary in order for arterial endothelial cells to avoid venous endothelial cells expressing EphB4 and ultimately reach their correct location, resulting in the formation of distinct arteries and veins.

6.5 A role for ephrin-B2 signalling in cancer progression

Eph/ephrin signalling is well documented to have a role in tumourigenesis. The main function of Eph/ephrin signalling seems to be during tumour angiogenesis and growth (Surawska et al., 2004). Ephrin-B2 has previously been implicated in tumourigenesis (Nikolova et al., 1998; Noren et al., 2004) and the results I have presented here would fit with the cell morphologies described for cells during tumour invasion. Two different modes of tumour-cell motility through a 3D matrix have been reported recently (Sahai and Marshall, 2003; Wilkinson et al., 2005). Rho signalling through ROCK promotes rounded cell motility, with cells exhibiting a rounded morphology and associated membrane blebs. An elongated mode of motility has also been described which functions independently of Rho but is associated with Rac-dependent actin-rich protrusions (Sahai and Marshall, 2003). There are parallels between the morphological changes observed after ephrin-B2 activation in HUAECs and both the rounded and elongated modes of tumour-cell motility. Initially, EphB4 triggers cell retraction or rounding, and in some cells membrane blebbing, which is dependent on Rho and ROCK activity and can be favourably compared to the first example of tumour-cell motility described above. Subsequently the cells recover and re-spread large lamellae, which are known to be Rac-dependent structures (Nobes and Hall, 1995), and at the onset of recovery there is a decrease in Rho and ROCK activity suggesting they are not required for re-spreading. Rac activity throughout the time course of EphB4 stimulation has not been investigated but it would be interesting to determine if there is an increase in

Rac activity at the onset of recovery. Very recently the elongated mode of motility has actually been reported to require Cdc42-MRCK (myotonic dystrophy kinase-related Cdc42-binding kinase) signalling (Wilkinson et al., 2005). However, the activity of Cdc42 across the EphB4 treatment time course in HUAECs is not known.

Figure 6.1 Model of ephrin-B signalling triggered by EphB stimulation



In HUAECs, endogenous ephrin-B2 signalling leads to actomyosin contractility resulting in cell retraction dependent on JNK, Rho and ROCK activity. Retraction is transient, followed by re-spreading coincident with a down-regulation of Rho-GTP and ROCK. In Swiss 3T3 fibroblasts, clutsering of exogenous ephrin-B2 leads to loss of cell-cell contact dependent on serum factors and Grb4. Tyrosine phosphorylation of ephrin-B2 is necessary for a full response.

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